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**DEVELOPMENT OF SEPARATION METHODS
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YUKIHIRO ESAKA

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INTRODUCTION

Many kinds of separation methods have greatly contributed to advances in science and the advances have also stimulated demands for further developments of the separation methods. Recent separation techniques have frequently performed determination of a variety of analytes and also sequence analyses of biological macromolecules such as proteins and DNAs, and polysaccharides. On the other hand, isolation of important substances from complex matrices also essential procedure in detailed analysis of the structures and functions. For such purposes also, several separation methods have been widely employed.

Liquid chromatography (LC) and electrophoresis have been frequently employed for determination and isolation of biological substances. LC has been the most popular technique for such purposes because of (i) a variety of the separation modes involving ordinary phase, reversed phase, ion exchange, and gel permeation.; (ii) the ease in fractionation of analytes, and (iii) the broad applicability to non-volatile biological analytes. In addition, the development of high performance liquid chromatography (HPLC) systems has accelerated its availability. On the other hand, electrophoresis has an advantage in principle in separation of "ionic" substances and has given full play to its performance in separations of proteins, DNAs, and those fragments. In recent years, capillary electrophoresis (CE) has been developed and its excellent power has been demonstrated in the separation of compounds with wide range of molecular weights, as evidenced in zone electrophoresis, gel electrophoresis and isoelectrophoresis. In addition to these separation techniques, electrokinetic chromatography (EKC) has been developed as one of important separation mode in CE. The principle of the separation technique is characteristic of LC in part. Micellar electrokinetic chromatography (MEKC) is a popular EKC and exhibits the reversed phase mode. In contrast to LC, the interactions which control the separation in CE occur in homogeneous systems. This situation allows us to understand what is happening during separation.. This means that CE is also devoted to basic and qualitative studies of separation modes. The resultant

knowledge is also expected to be reduced again to detailed understanding of LC separation.

LC and electrophoresis continue to be essential for separation analyses of biological fields and should be used complementary with each other. The purposes of this work are developments of separation methods in CE and LC and their application desiring chemical speciation of analytes and environmental analysis.

In **CHAPTER 1**, the author has focused his attention to hydrogen-bonding interaction as a new separation mode of CE. In CE, there exist three major modes for separation. They are called capillary zone electrophoresis (CZE), electrokinetic chromatography and capillary gel electrophoresis. Development of a new separation mode in capillary electrophoresis will greatly extend availability of CE. One of possible interactions to be utilized for separation would be the electrostatic interactions. Such CE based on an electrostatic interaction mode will be useful for separation of a broad spectrum of biological molecules possessing various functional groups.

Several compounds with polyether structure are known to interact with various substances by their electron donor activities, as observed in inclusion phenomena of crown ethers and poly (ethylene glycol) toward cations. Considering these phenomena, the author has expected hydrogen-bonding activity of polyethers and studied to establish a new separation mode based on hydrogen-bonding interactions between polyethers and solutes in CE. Using PEG as a separation matrix in CZE, the interaction has been analyzed quantitatively. In order to enhance the interaction in CE, the author has utilized mixed micellar systems composed of polyether type surfactants, Tween 20 or Brij 35, and sodium lauryl sulfate, which provides hydrophobic circumstances to analytes. The improved separation has been realized for benzene derivatives used as model analytes. The distribution between the micelle and aqueous phases and then the separation is theoretically discussed from the thermodynamic point of view.

In **CHAPTER 2**, chromatofocusing (CF) has been examined to demonstrate as a method for accurate separation analysis of proteins. Proteins are generally considered to have several analogues such as isomers, mutants, and post-translational derivatives in biological tissues. Employment of multi-dimensional

separation techniques will be useful for purification and identification of interest proteins. The author has focused on CF, a LC methods having isoelectrophoresis-like separation selectivity, using commercially available sperm whale myoglobin as model analytes, which is reported to contain several isomers of myoglobin. Components separated by CF has been characterized in view of molecular weights, iron contents, and UV-vis spectra by means of size exclusion chromatography, atomic absorption spectrometry, and photodiode array detection, respectively.

CHAPTER 3 involves the development of analytical methods for determination and identification of the redox cofactor pyrroloquinoline quinone (PQQ) and its application from the viewpoints of the chemical speciation of PQQ in biological media. PQQ has been discovered from methylotrophs as a redox cofactor of methanol dehydrogenase and its free form was been expected to be widespread in mammals and foods made from them. However the detection of PQQ in biological matrices is difficult because of its low concentration and its high reactivity toward nucleophilic reagents leading to corresponding derivatives. As a results, PQQ has not been detected certainly in foods, mammalian fluids, etc., which were believed to contain PQQ. On the other hand, much attention has been attracted by several pharmaceutical activities of PQQ, for example, a therapeutic effect on cataract and a stimulative effect to produce nerve growth factor (NGF) which is considered as a potent-drug for Alzheimer's syndrome. In these situations, development of detection methods of PQQ with high selectivity and high sensitivity are required.

The author has studied a high-performance liquid chromatographic (HPLC) determination of PQQ with an electrochemical detector coupled with a redox-cycling reaction of free PQQ by using ferricyanide ion ($\text{Fe}(\text{CN})_6^{3-}$). The chemical amplification has successfully enhanced the sensitivity. On the other hand, we have employed CE for determination of oxazolopyrroloquinoline (OPQ), PQQ derivatives from α -amino acids, which are potent forms of PQQ in biological media. The OPQs are strongly charged molecules owing to the three carboxyl groups and also to the acidic and basic amino acid residues. Additionally their structures are only slightly different from each other. These situations make

difficult for usual LC methods to separate all OPQs. In contrast, CE has excellent power in separation of charged solutes. Detailed examination of the separation conditions has realized almost complete separation of OPQs. The reaction between PQQ and α -amino acids and the kinetics have been investigated by CE. Several biological samples has been also analyzed by CE and the forms of PQQ in the biological media is discussed.

In **CHAPTER 4**, development of a detection method of alkaline phosphatase (APase) in natural water is presented. It is well known that natural water exhibits APase activity and it is also suggested that APase derived from bacteria controls phosphorus cycles of natural water on the globe.

In this study, a detection method based on enzymatic reaction of APase with 4-methylumbelliferyl phosphate as a fluorometric reagent has been combined with SEC separation, in order to realize highly selective and sensitive on-line detection of APase. In principle, the SEC separation gives us the information concerning molecular weights of APases, which is useful for us to identify their origin. Using this method, we have determined APase activity of pond water.

CHAPTER 1

DEVELOPMENT OF CAPILLARY ELECTROPHORESIS WITH HYDROGEN-BONDING MODE USING POLYETHERS

1.1 Capillary Electrophoresis using Polyethers as Matrix with hydrogen-bonding activity

Electrostatic interaction between polyethers and various substances continues to be a topic of interest in several fields. Crown ethers are well known to possess the metal ion recognition ability.¹⁻⁸ On the other hand, Triton X-100, a surfactant with a non-cyclic polyether moiety, has been reported to roll alkaline metal ions like "turban" with its polyether chain in organic phases.⁹ Recent studies on poly(ethylene oxide) (PEO) solutions of alkaline metal salts have revealed that the ion-dipole interaction between metal ions and the ether oxygen of PEO significantly affects the ionic transfer.^{10,11} Hydrogen bond is also electrostatic in nature and is observed in interaction between polyethers and some hydrogen donors. An example is the interaction between Lasalocid A (a non-cyclic polyether ionophore) and amine complexes such as $[\text{Co}(\text{NH}_3)_6]^{3+}$, which leads to corresponding adduct formation involving hydrogen bond in hydrophobic media.^{12,13}

In capillary electrophoresis, there exist three major modes for separation. They are called capillary zone electrophoresis (CZE), electrokinetic chromatography and capillary gel electrophoresis. Development of a new separation mode in capillary electrophoresis will greatly extend availability of capillary electrophoresis. One of possible interactions to be utilized for separation would be the electrostatic interactions mentioned above. Although hydrogen bond is relatively weak in strength, it is frequently encountered for organic compounds. In this paper, we attempt to utilize the hydrogen bonding ability of PEG in capillary electrophoresis as a general and new separation mode.

EXPERIMENTAL

Three kinds of poly(ethylene glycol) (PEG) with mean molecular weights of 400, 4000, and 20000 (PEG 400, PEG 4000, PEG 20000) were obtained from Kishida Chemical (Osaka, Japan) and used as received. *p*-Acetamidebenzoic acid, acetylsalicylic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, *o*-phthalaldehydeic acid, salicylic acid, benzoic acid, adenosine 5'-monophosphoric acid (5'-AMP), guanosine 5'-monophosphoric acid (5'-GMP), cytidine 5'-monophosphoric acid (5'-CMP), and thymine 5'-monophosphoric acid (5'-TMP) were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

Apparatus

Electrophoretic separation was performed in a fused silica tubing (GL Science, Tokyo, Japan) with 0.05-mm i.d. and a column length of 750 mm. In this column 500 mm was the effective length for separation. A Matsusada Precision Devices Model HCZE-30PNO high-voltage dc power supply (Kusatu, Japan) which can deliver high voltage up to 30 kV was employed to supply the potential across the capillary. For UV-VIS spectrophotometric detection, a Jasco 875-UV detector (Tokyo, Japan) was employed. The UV spectrophotometric detection was done at the negative potential side. The detection wavelength was set at 210 nm and 254 nm for the benzoic acids and the nucleotides, respectively. A flow cell was modified for capillary electrophoresis in our laboratory. A Shimadzu Chromatopac C-R6A (Kyoto, Japan) was used for data processing.

Procedure

Just before each run, a separation capillary tube was purged with 0.1 M NaOH for 2 min and then with a desired buffer for 3 min by using an aspirator. The two ends of the tube were then dipped, respectively, into two separated 1.5-ml reservoirs filled with the same buffer. The end at which samples were introduced was connected with a platinum electrode to the positive high voltage, while the other end was connected with a platinum electrode to ground. Samples were

introduced by siphoning at a height of 15 cm for a 5 to 10-s period. Other details were described in our previous paper.¹⁴

RESULTS AND DISCUSSION

Migration behaviors of substituted and unsubstituted benzoic acids

PEG 400 was added at concentrations ranging from 1 to 20% (v/v) to an electrolyte solution of 10 mM phosphate buffer, pH 7.8. Because of the increased viscosity of PEG with higher molecular weights, the maximum concentration was set as 10% (v/v) for PEG 4000 and 5% (v/v) for PEG 20000. Six substituted benzoic acids, *p*-acetamidebenzoic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, salicylic acid, acetylsalicylic acid, and *o*-phthalaldehydeic acid, and benzoic acid were used as model samples. Each of the former four compounds has a substituent with donor-property in the hydrogen-bonding: amide, amino, and hydroxyl groups in *para* or *ortho* position, respectively, while the others are inactive as hydrogen donor. All these compounds are reasonably considered as univalent anions under the present conditions, because the common carboxyl group is completely deprotonated.

Figure 1 shows electropherograms of the seven benzoic acids in the absence (A) and presence of 5% (v/v) of PEG 400 (B). In the absence of PEG, the separation is attributable mainly to the difference in the effective hydrodynamic size involving solvation and then it was not complete. Improved separation was achieved by the PEG addition, especially for *p*-hydroxybenzoic acid (peak 3), *p*-aminobenzoic acid (peak 4), and *o*-phthalaldehydeic acid (peak 5), although the net migration times increased due to the elevated viscosity. Pronounced influence of the PEG addition is a decrease in the relative migration times of *p*-acetamidebenzoic acid (peak 1), *p*-hydroxybenzoic acid (peak 3), *p*-aminobenzoic acid (peak 4), and salicylic acid (peak 7) compared with that of *o*-phthalaldehydeic acid (peak 5). Occurrence of some attractive interaction of an analyte anion with PEG should decrease the relative electrophoretic velocity in the positive potential direction because PEG migrates in the negative potential direction at the electroosmotic flow rate (V_{EO}), and then it accelerates the relative mobility of the analyte in the negative

potential direction. Therefore, the experimental result indicates stronger attractive interaction with PEG of *p*-acetamidebenzoic acid, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid compared with *o*-phthalaldehydeic acid.

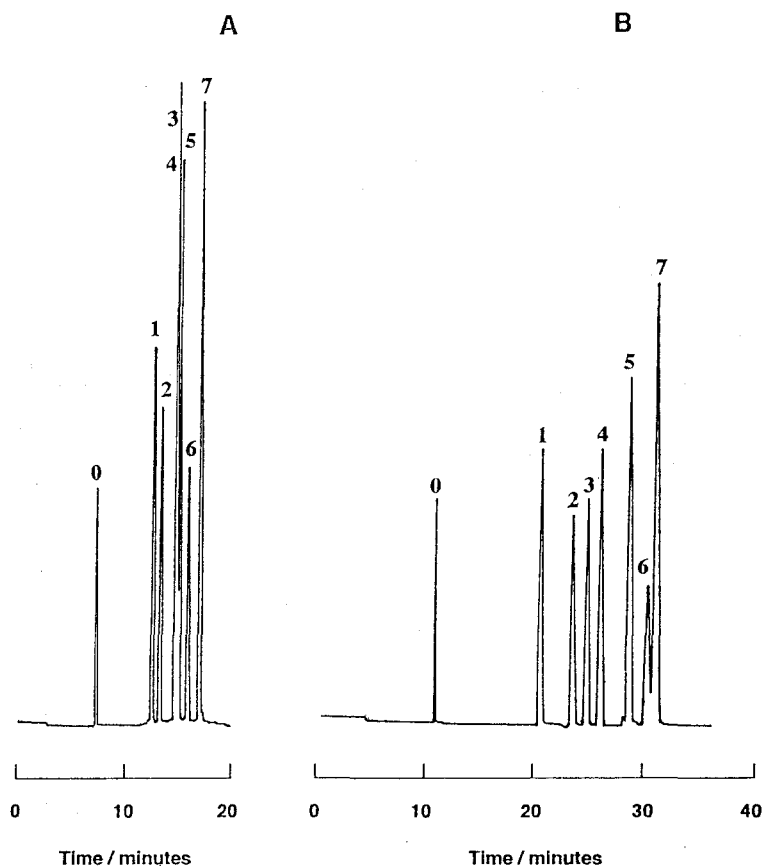


Figure 1 Electropherograms of seven substituted and unsubstituted benzoic acids in the absence of PEG (A) and presence of 5 % (v/v) PEG 400 (B) in electrolyte solution.

Electrolyte solution, 10 mM phosphate buffer (pH 7.8); capillary, 750 mm 0.05 mm i.d. (500 mm effective length); applied voltage (current) , 11 kV (5 A) in both of (A) and (B); detection wavelength, 210 nm. Peak : (0) mesityl oxide (an electroosmotic flow marker), (1) *p*-acetamidebenzoic acid, (2) acetylsalicylic acid, (3) *p*-hydroxybenzoic acid, (4) *p*-aminobenzoic acid, (5) *o*-phthalaldehydeic acid, (6) benzoic acid, and (7) salicylic acid.

It was reported that PEG with its molecular weight of 40000 exhibits a molecular sieving effect in conventional electrophoresis of proteins.¹⁵ However, such an effect makes a minor contribution, if any, in our case, because a lower molecular weight PEG 4000 showed no effect on the electrophoretic separation of proteins¹⁵ and the molecular weights of the analytes used here are low and close to one another. Therefore, the observed effect of the PEG addition in the capillary electrophoresis is reasonably attributable to the hydrogen bond formation between the ether moieties of PEG and the amide group of *p*-acetamidebenzoic acid, the amino group of *p*-aminobenzoic acid, or the hydroxyl groups of *p*-hydroxybenzoic acid and salicylic acid.

Evaluation of the interaction between analytes and poly(ethylene glycol) (PEG)

Here let us consider that analyte anion is in equilibrium between two states free from and bound to PEG. Assuming stoichiometric interaction between the analyte and PEG, the hydrogen-bonding complex formation constant (K) is given by

$$K = x/(1 - x)[\text{PEG}] \quad (1)$$

where x denotes the fraction of the analyte bound to PEG. The quantity $[\text{PEG}]$ is the concentration of PEG (or of the polyether segment). Under these conditions, the electrophoretic velocity of the analyte ion (V_{ep}) is expressed as^{16,17}

$$V_{\text{ep}} = (1 - x)V_{\text{ep,f}} + xV_{\text{ep,c}} \quad (2)$$

where $V_{\text{ep,f}}$ and $V_{\text{ep,c}}$ are the electrophoretic velocity of the free analyte ion and that of the analyte-PEG hydrogen-bonding complex. When $K[\text{PEG}] \ll 1$, eqs 1 and 2 can be reduced to

$$V_{\text{ep}} = V_{\text{ep,f}} + K(V_{\text{ep,c}} - V_{\text{ep,f}})[\text{PEG}] \quad (3)$$

Increase in the PEG concentration affects the viscosity of the electrophoretic medium (and then $V_{\text{ep,f}}$ and $V_{\text{ep,c}}$) as well as x . In order to eliminate such effect arising from viscosity change, we consider a relative value of V_{ep} against the electrophoretic velocity of a reference compound with $K \approx 0$ ($V_{\text{ep,o}}$) under given electrophoretic conditions. Thus $V_{\text{ep}}/V_{\text{ep,o}}$ is given as

$$V_{ep}/V_{ep,o} = V_{ep,f}/V_{ep,o} + K[(V_{ep,c} - V_{ep,f})/V_{ep,o}][PEG] \quad (4)$$

In the present analysis, *o*-phthalaldehydeic acid was chosen as a reference because its specific interaction with PEG appears to be negligible, as mentioned above.

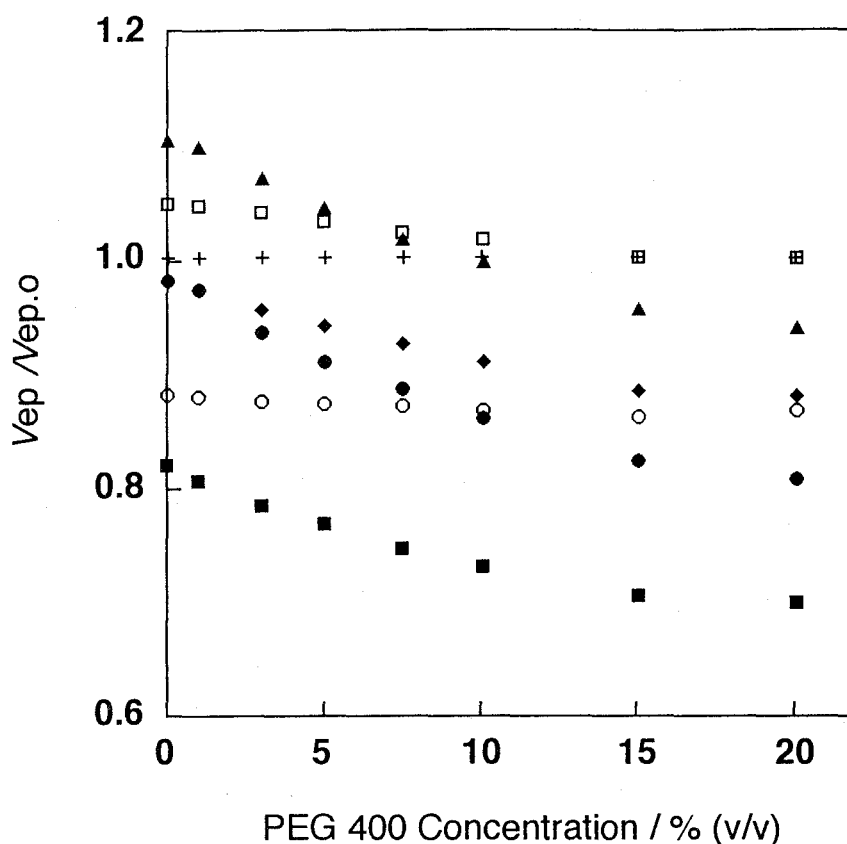


Figure 2 Dependence of relative electrophoretic velocity ($V_{ep}/V_{ep,o}$) of the benzoic acids on the concentration of PEG 400:

(■) *p*-acetamidebenzoic acid, (●) *p*-hydroxybenzoic acid, (◆) *p*-aminobenzoic acid, (▲) salicylic acid, (□) benzoic acid, (○) acetylsalicylic acid, (+) *o*-phthalaldehydeic acid.

Figure 2 shows dependence of $V_{ep}/V_{ep,o}$ on the concentration of PEG 400. In this study, the values of V_{ep} were estimated based on its relationship with the net (measured) migration velocity (V) of an analyte:

$$V = V_{ep} + V_{eo} \quad (5)$$

where V_{eo} (electroosmotic velocity) has the opposite sign (plus) to V_{ep} of negatively charged analytes. The values of V_{eo} were evaluated from the migration time of a neutral marker, mesityl oxide, which was co-injected with the samples. For *p*-acetamidebenzoic acid, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid, their $V_{ep}/V_{ep,o}$ values go down linearly with [PEG] at low [PEG] and then level off at increased [PEG]. The linear relationship is well described by eq 4. The negative slope is due to the fact that $V_{ep,c} \ll V_{ep,f}$. The leveling-off dependence at high [PEG] can be attributable to saturation phenomena as predicted from eq 1. In contrast, $V_{ep}/V_{ep,o}$ of acetylsalicylic acid is almost independent of [PEG]. The best resolution was achieved at [PEG] 3% (v/v) as judged from Fig. 2.

Similar plots were observed for PEG 4000 and PEG 20000. The linear parts of the $V_{ep}/V_{ep,o}$ vs. [PEG] plots at low [PEG] (1 - 5 % (v/v)) allow the estimation of $K[(V_{ep,c}-V_{ep,f})/V_{ep,o}]$ values on the basis of eq 4, as listed in the parentheses of Table 1. Here let us assume that $V_{ep,c} - V_{ep,f} \approx -V_{ep,f}$ because PEG itself is neutral. This would be true for PEG with sufficiently larger hydrodynamic size (or larger molecular weight) such as PEG 4000 or PEG 20000 compared with that of free analyte anions (see later). Under this assumption, values of K are easily estimated from the slope and intercept of the linear plots of $V_{ep}/V_{ep,o}$ against [PEG]. Those values are summarized in Table 1. When the K values of the analytes are compared with one another, the interaction with PEG can be concluded to decrease in order of *p*-hydroxybenzoic acid *p*-acetamidebenzoic acid, salicylic acid, *p*-aminobenzoic acid, and benzoic acid for all of PEGs used. Acetylsalicylic acid as well as *o*-phthalaldehydeic acid does not seem to interact with PEG. This result can be fundamentally interpreted in terms of the hydrogen-bonding interaction. Phenolic hydroxyl group serves as a good hydrogen donor. This is what we can see for *p*-hydroxybenzoic acid and salicylic acid. In the latter

case, however, the intramolecular hydrogen bond formation seems to compete with the interaction with PEG, resulting in a reduction of K compared with that of the former. The amide group of *p*-acetamidebenzoic acid is stronger in acidity and then in hydrogen-donating propensity than the amino group of *p*-aminobenzoic acid. The acetoxy group of acetylsalicylic acid and the formyl group of *o*-phthalaldehydeic acid are inactive as hydrogen donor. On the other hand, the K value of benzoic acid is larger than that of acetylsalicylic acid, respectively. This result would not be simply interpreted in terms of the hydrogen-bonding interaction and then suggests the occurrence of some additional weak interaction, most probably, hydrophobic interaction. Such hydrophobic interaction with other nonionic polymers has been reported by Hjerten et al.¹⁸

Table 1 Estimated values of K

	$K \times 10^3 / [\text{PEG}]^{-1} \text{ a, b}$		
	PEG 400	PEG 4000	PEG 20000
<i>p</i> -Hydroxybenzoic acid	16.1(-15.8)	19.2(-19.1)	19.9(-19.5)
<i>p</i> -Acetamidebenzoic acid	11.4 (-9.2)	19.3(-15.7)	19.3(-15.7)
Salicylic acid	12.0(-13.2)	12.9(-14.2)	18.6(-20.5)
<i>p</i> -Aminobenzoic acid	8.2 (-8.0)	10.3(-10.1)	10.3(-10.1)
Benzoic acid	3.6 (-3.8)	8.0 (-8.5)	6.7 (-7.1)
Acetylsalicylic acid	1.4 (-1.2)	3.8 (-3.3)	3.4 (-3.0)

^a [PEG] in % (v/v).

^b Values in the parentheses represent the slopes of $V_{\text{ep}}/V_{\text{ep},0}$ vs. [PEG] plots (see Fig. 2) in $10^{-3} [\text{PEG}]^{-1}$, which correspond to $K(V_{\text{ep},c} - V_{\text{ep},f})/V_{\text{ep},0}$ (see eq 4).

Table 1 indicates also the significance of the polymer chain length. The K values of PEG 4000 is larger than that of PEG 400 for all analytes used. However, the those of PEG 4000 and PEG 20000 are comparable with each other. This result can be interpreted as follows. It is known that polymer chains in solution are entangled at increased concentrations. The critical concentration (Φ) is calculated as about 16, 2.5, and 0.7 % (v/v) for PEG 400, 4000, and 20000, respectively, by using $\Phi = N^{-0.8}$, where N is the number of segments in the polymer chain.¹⁹ Thus, PEG 4000 and PEG 20000 can be considered to exist as entangled polymers, while PEG 400 is isolated from one another at relatively low concentration. Therefore, it is supposed to be that $V_{ep,c} - V_{ep,f} < V_{ep,f}$ for PEG 400. In the above estimation of K in Table 1, however, we have used the assumption that $V_{ep,c} - V_{ep,f} \approx -V_{ep,f}$, which leads to underestimation of K for PEG 400.

Another interpretation would be possible as follows. The analyte as a hydrogen donor may migrate along the PEG chain to interact with the neighboring ether segments consecutively. As a result, the increase in the chain length of PEG will increase an apparent collision probability and then enhance the net interaction. However, such migration along the chain may be continued only up to several-ten segments, which is larger than the chain length of PEG 400 and shorter than that of PEG 4000. Therefore, further increase in the chain length over PEG 4000 brings about no additional increase in the net interaction.

Application of the hydrogen-bonding mode CE in separation of nucleotides

The present hydrogen bonding mode is expected to work well in CZE separation of nucleotides, of which the hydrogen-bonding ability has been well emphasized in biochemistry. We studied effect of PEG addition on the separation of four nucleotides, 5'-GMP, 5'-AMP, 5'-TMP, and 5'-CMP.

In the absence of PEG, the separation only between the purine nucleotides (5'-GMP and 5'-AMP) and pyrimidine nucleotides (5'-TMP and 5'-CMP) was observed as shown in Fig. 3(A). As preliminary study, four bases, guanine, adenine, thymine, and cytosine, were subjected to CZE. All these bases migrated

simultaneously at the electroosmotic flow rate, indicating that the bases are not charged under the present conditions (pH 7.8). This result suggests that the four nucleotides used are identically divalent because of the acid dissociation of the common phosphate group. Therefore, the separation can be attributable to the difference in the hydrodynamic sizes of purine and pyrimidine moieties.

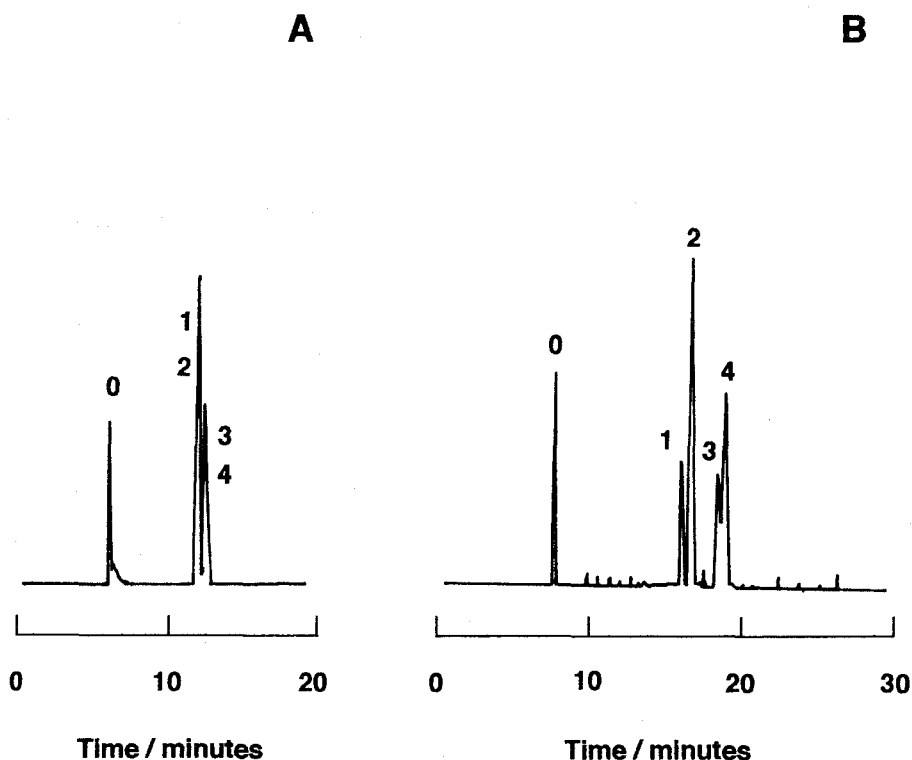


Figure 3. Electropherograms of four nucleotides in the absence of PEG (A) and presence of 10 % (v/v) PEG 4000 (B) in electrolyte solution.

Electrolyte solution, 10 mM phosphate buffer (pH 7.8); capillary, 750 mm \times 0.05 mm i.d. (500 mm effective length), applied voltage (current) 10 kV (6 A) and 14 kV (5 A) in (A) and (B), respectively; detection wavelength, 254 nm. Peaks 0-4: mesityl oxide (an electroosmotic flow marker), 5'-GMP, 5'-AMP, 5'-TMP, and 5'-CMP, in that order.

Addition of 10% (v/v) of PEG 4000 into the electrophoretic medium improved the separation drastically as shown in Fig. 3(B). The improved separation seems to be caused by the hydrogen-bonding interaction with PEG. The amide and amino groups in these nucleotides are likely to function as hydrogen donors against the ether segment of PEG. The net electrophoretic mobility was in order of 5'-GMP, 5'-AMP, 5'-TMP, and 5'-CMP. The stronger interaction of 5'-GMP compared with 5'-AMP would be interpreted by the fact that 5'-GMP has two hydrogen donor sites at N(1) and C(2) positions, while 5'-AMP has only one site at C(6) position. On the other hand, the slightly stronger interaction of 5'-TMP compared with 5'-CMP would be arisen from the acidity of the amide group in 5'-TMP compared with that of the amino group in 5'-CMP. The latter consideration is supported by the fact that *p*-acetamidebenzoic acid interacts with PEG much strongly than *p*-aminobenzoic acid.

Akashi and colleagues reported capillary gel electrophoretic separation of three oligodeoxynucleotide isomers consisting of five thymidylic acids and one adenylic acid, TpTpTpApTpT, TpTpTpTpApT, and TpTpTpTpTpA, with poly(9-vinyladenine)-entrapped polyacrylamide gel.^{20,21} They have concluded that the base-pair formation between thymine and poly(9-vinyladenine) plays an important role in the separation and termed it capillary "affinity" gel electrophoresis. Such hydrogen-bonding interaction is supposed to work in capillary electrophoresis using cyclodextrins²²⁻²⁴ and also in isotachophoresis using PEG as additive.²⁵

APPENDIX

In the above formulation, PEG has been considered as a carrier, as in the case of electrokinetic chromatography^{16,17} (see eq 2) and stoichiometric interaction between an analyte and PEG is assumed (see eq 1). Another model of this separation mode is that PEG works as a matrix in electrophoretic medium. Using this model, the effect of the PEG addition can be described as follows.

In capillary zone electrophoresis, the electrophoretic velocity (V_{ep}) is related to the applied electric field (E) by²⁷

$$fV_{ep} = qE \quad (A1)$$

where q is the charge of an analyte and f is called the translational friction coefficient. The effect of the PEG addition on the migration can be attributable to the relative change in f . If we assume spherical shape for the analytes used, f is given by Stokes' law as $f = 6\pi a\eta$ in the absence of PEG, where a and η are the apparent hydrodynamic radius of the analyte and the viscosity of the electrophoretic medium, respectively. When some attractive interaction occurs in the presence of PEG, it brings about additional friction. The increase in [PEG] causes an increase in number of collision between PEG and a solute, which leads to an increase in the additional friction. Therefore f is given by

$$f = 6\pi a\eta + k[\text{PEG}] \quad (A2)$$

where k is a constant representing the strength of the interaction. When a reference compound which does not specifically interact with PEG, the friction can be simply expressed by

$$f_0 = 6\pi a_0\eta \quad (A3)$$

Combination of eqs A2 and A3 yields

$$f/f_0 = a/a_0 + (k/f_0)[\text{PEG}] \quad (A4)$$

Experimentally estimated f/f_0 increased linearly with [PEG] at low [PEG] and then became to level off at higher [PEG] for all analytes. The [PEG] dependence is very similar to that in Fig. 2, though the slope has the opposite sign to each other. The linear dependence was well described by eq A4. Values of k evaluated using eq A4 showed such dependencies on analytes and/or PEGs as the K values in Table 1 have.

SUMMARY

Addition of poly(ethylene glycol) (PEG) as a free matrix was found to be a novel separation parameter in capillary zone electrophoresis. In the separation of substituted and unsubstituted benzoic acids used as model analytes, attractive interaction with PEG was observed for the analytes with hydroxyl, amide, or amine groups. This interaction is attributable to hydrogen bond formation. The concentration and the polymer length of PEG can control the net strength of the interaction and then the observed separation. This capillary electrophoresis using the hydrogen-bonding mode was successfully applied to the separation of nucleotides.

REFERENCES

- (1) C. J. Pedersen, *J. Am. Chem. Soc.*, 89 (1967) 7017.
- (2) C. J. Pedersen, *J. Am. Chem. Soc.*, 92 (1970) 386.
- (3) H. K. Frensdorff, *J. Am. Chem. Soc.*, 93 (1971) 600.
- (4) H. K. Frensdorff, *J. Am. Chem. Soc.*, 93 (1971) 4684.
- (5) T. Takeda and H. Goto, *Bull. Chem. Soc. Jpn.*, 52 (1979) 1920.
- (6) T. Takeda, *Bull. Chem. Soc. Jpn.*, 53 (1980) 2393.
- (7) T. Takeda and Y. Matsumoto, *Bull. Chem. Soc. Jpn.*, 60 (1987) 2313.
- (8) T. Takeda, Y. Kimura, Y. Kudo, Y.; Matsuda, H. *Bull. Chem. Soc. Jpn.*, 62 (1989) 2885.
- (9) Y. Kikuchi, N. Takahashi, T. Suzuki and K. Sawada, *Anal. Chim. Acta*, 256 (1992) 311.
- (10) H. Ohno, N. Kobayashi, S. Takeoka, H. Ishizaka and E. Tsuchida, *Solid State Ionics*, 40/41 (1990) 655.
- (11) H. Ohno and P. Wang, *Nippon Kagaku Kaishi*, 12 (1991) 1588.
- (12) L. F. Lindoy and G. W. Walker, *J. Am. Chem. Soc.*, 112 (1990) 3659.
- (13) P. S. K. Chia, L. F. Lindoy, G. W. Walker and G. W. Evertt, *J. Am. Chem. Soc.*, 113 (1991) 2533.
- (14) Y. Esaka, Y. Yamaguchi, K. Kano and M. Goto, *J. Chromatogr. A*, 652 (1993) 225.
- (15) H. -J. Bode, *FEBS Lett.* 65 (1976) 56.
- (16) K. Otsuka, S. Terabe and T. Ando, *J. Chromatogr.* 332 (1985) 219.
- (17) S. Terabe and T. Isemura, *Anal. Chem.* 62, (1990) 650-652.
- (18) S. Hjerten, L. Valtcheva, K. Elenbring and D. Eaker, *J. Liq. Chromatogr.* 12 (1989) 2471.
- (19) P. D. Grossman, In *Capillary Electrophoresis, Theory and Practice*; P. D. Grossman and J. C. Colburn (Eds.), Academic Press, 1992, pp. 215-233.
- (20) M. Akashi, T. Sawa, Y. Baba and M. Tsuhako, *J. High Resol. Chromatogr.* 15 (1992) 625.
- (21) Y. Baba, M. Tsuhako, T. Sawa, M. Akashi and E. Yashima, *Anal. Chem.* 64 (1992) 1920.

- (22) A. Guttman, A. Paulus, A. S. Cohen, N. Grinberg and B. J. Karger, *J. Chromatogr.* 448 (1988) 41.
- (23) I. D. Cruzado and G. Vigh, *J. Chromatogr.* 608 (1992) 421.
- (24) H. Nishi, T. Fukuyama and S. Terabe, *J. Chromatogr.* 553 (1991) 503.
- (25) P. Praus and V. Dombek, *Anal. Chim. Acta*, 283 (1993) 917.
- (26) B. J. Herrin, S. G. Shafer, J. van Alstine, J. M. Harris and R. S. Snyder, *J. Colloid. Interfac. Sci.* 115 (1987) 46.
- (27) P. D. Grossman, In *Capillary Electrophoresis, Theory and Practice*; P. D. Grossman and J. C. Colburn (Eds.), Academic Press, 1992, pp. 111-120.

1.2 Hydrogen-Bonding Interaction in Capillary Electrophoresis Using Polyether Matrices

Increasing attention has been paid to the use of capillary electrophoresis (CE) in the analysis of biological and pharmaceutical fields.¹⁻³ In CE, capillary zone electrophoresis (CZE), electrokinetic chromatography, and capillary gel electrophoresis are generally considered to be the three modes of separation. Each of the modes has been realizing characteristic separation of a variety of samples. Development of a new separation mode is much in demand for further extension of the availability of CE.

One of possible interaction to be utilized for separation would be electrostatic interaction. With this point in mind we focused our attention on polyethers, which are known to serve as electrostatic electron donors via their ether oxygen atoms. Typical examples are inclusion phenomena of cations by crown ethers and non-cyclic polyethers.⁴⁻¹² Noteworthy, hydrogen bonding is also observed between a polyether ionophore and amine complexes in hydrophobic media.^{13,14} These studies have stimulated us to utilize the hydrogen-bonding ability of polyethers to develop a new "hydrogen-bonding mode" or "electrostatic mode" in CE.¹⁵

Our earlier study revealed that the addition of poly(ethylene glycol) (PEG) as a free matrix can greatly improve the CZE separation of benzoic acid derivatives used as model analytes as a result of the interaction between PEG and the analytes.¹⁵ The strength of the interaction is appropriate to control the mobility of the analytes and appears to depend on the hydrogen-donating activity of the substitutes. Thus we described the phenomenon in terms of hydrogen-bonding interaction between PEG and the analytes.

In the present study, we attempt to get further evidence for the hydrogen-bonding formation between PEG and analytes during the CZE separation. Our strategy is to investigate effects of temperature of capillary and urea as an additive on the strength of the interaction between PEG and analytes. It is the reason that hydrogen bonding is weakened at elevated temperature and that urea, bifunctional

hydrogen donor and acceptor, breaks hydrogen-bonding complexes as a result of hydrogen-bonding exchange. Furthermore, nuclear magnetic resonance (NMR) spectroscopy would provide direct evidence of hydrogen-bonding complex formation. We will also discuss some other minor interactions controlling the separation.

EXPERIMENTAL

Three kinds of poly(ethylene glycol) (PEG) with mean molecular weights of 400, 4000, and 20000 (PEG 400, PEG 4000, PEG 20000) were obtained from Kishida Chemical (Osaka, Japan) and used as received. 4-Acetamidobenzoic acid (4CH₃CONH-BA), 4-acetoxybenzoic acid (4CH₃COO-BA), 4-aminobenzoic acid (4NH₂-BA), 4-hydroxybenzoic acid (4OH-BA), 4-methylbenzoic acid (4CH₃-BA), 4-carboxybenzaldehyde (4CHO-BA), 2-hydroxybenzoic acid (2OH-BA), 2-carboxybenzaldehyde (2CHO-BA), benzoic acid (BA) and chloroform-D (CDCl₃, containing 1% TMS) were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

Electrophoretic separation was performed in a fused silica tubing (GL Science, Tokyo, Japan) with 0.05-mm i.d. and a column length of 750 mm. In this column 500 mm was the effective length for separation. Samples were introduced at the end to be connected to the positive high voltage by siphoning at a height of 15 cm usually for a 5 to 10-s period. When PEG was used at higher concentrations, longer injection time was required in the sample injection, because of increased viscosity. Thermal control of the column was performed as follows. About 60 % of the effective length of the column was passed through a silicon tubing with 1.0 mm i.d. covered with a heat insulating material and then water controlled at a given temperature was pumped with a peristaltic pump (Gilson Minipuls 2, ca. 2 ml min⁻¹) through the silicon tubing during separations in the direction opposite to the electroosmotic flow. The UV spectrophotometric detection was done at the negative potential side. The detection wavelength was set at 210 nm. Other details were described in our previous papers.^{15,16}

A series of ^1H NMR measurements of benzoic acids at various concentrations of PEG 20000 was carried out in CDCl_3 with JEOL GX-270 operating at 270 MHz.

RESULTS AND DISCUSSION

Nine substituted and unsubstituted benzoic acids, $4\text{CH}_3\text{CONH-BA}$, $4\text{NH}_2\text{-BA}$, 4OH-BA , 2OH-BA , $4\text{CH}_3\text{COO-BA}$, 4CHO-BA , $4\text{CH}_3\text{-BA}$, 2CHO-BA , BA were used as model samples in CZE experiments. PEG 400 and PEG 4000 were used at concentrations ranging from 1 to 10 % in an electrolyte solution of 10 mM phosphate buffer (pH 7.8). Under the present separation conditions, all the analytes are considered as univalent anions because of complete dissociation of their carboxyl groups. As reported in our previous paper,¹⁵ the addition of PEG drastically influences the migration time of these benzoic acids. The influence can be described in terms of the following two factors. The first one is non-specific and is ascribed to the increase in the viscosity, resulting in the increase in the migration time. In the case of 2CHO-BA , the change in the migration time can be simply described by this effect, because it appears to exhibit no specific interaction with PEG (see later also).¹⁵ The second one, in which we are interested, is specific to analytes. The addition of PEG accelerates the mobility of some benzoic acids, especially four benzoic acids with the hydrogen-donating substituents ($4\text{CH}_3\text{CONH-BA}$, $4\text{NH}_2\text{-BA}$, 4OH-BA , 2OH-BA), compared with 2CHO-BA . The acceleration of the migration time is resulted from some attractive interaction between the hydrogen-donating benzoic acids and PEG, since PEG migrates in the direction of the negative potential at the electroosmotic flow rate (V_{EO}). In order to eliminate the first non-specific factor, we will describe electrophoretic behavior using relative values of the migration time and electrophoretic velocity against those of 2CHO-BA as a reference compound (see eq. 1 also).

Temperature Effect

Figure 1 shows electropherograms of the nine benzoic acids under thermostated conditions at 3 °C (A) and 80 °C (B) in the presence of 7.5 % (v/v) PEG 4000. Comparison of the two electropherograms reveals that the relative migration time of the four hydrogen-donating benzoic acids (4CH₃CONH-BA, 4NH₂-BA, 4OH-BA, 2OH-BA) increases at elevated temperature. This means that the raise in the column temperature weakens the interaction between PEG and the four benzoic acids having a hydrogen-donor active substituent. It is well known that an raise in temperature weakens and breaks hydrogen bonding. Thus, this behavior is in accord with our previous description in terms of the hydrogen-bonding complex formation between the hydrogen-donating benzoic acids and PEG.

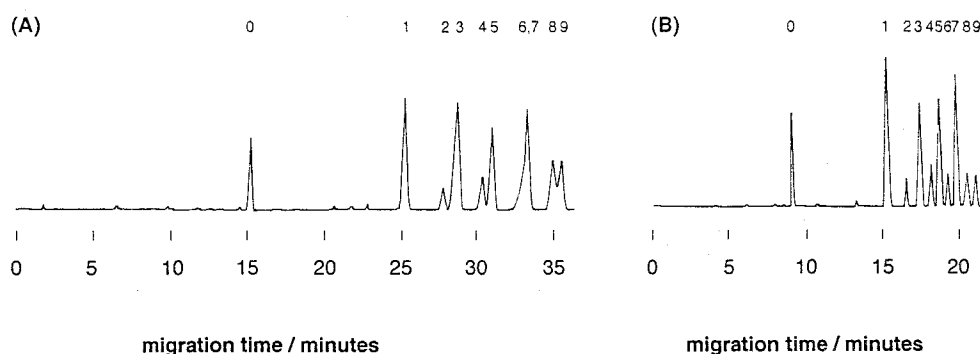


Figure 1 Electropherograms of nine substituted and unsubstituted benzoic acids with 7.5 % (v/v) PEG 4000 under the thermal control at (A) 3 °C and (B) 80 °C.

Electrolyte solution, 10 mM phosphate buffer (pH 7.8); capillary, 750 mm × 0.05 mm i.d. (500 mm effective length); applied voltage(current), 14 kV (4 μA) and 14 kV (6 μA) in (A) and (B), respectively; detection wavelength, 210 nm. Peaks: (0) mesityl oxide (an electroosmotic flow marker), (1) 4CH₃CONH-BA, (2) 4CH₃COO-BA, (3) 4OH-BA, (4) 4CH₃-BA, (5) 4NH₂-BA, (6) 4CHO-BA, (7) 2OH-BA, (8) 2CHO-BA and (9) BA.

When we can assume a stoichiometric and equilibrated complex formation between an analyte and PEG, the observed electrophoretic velocity of the analyte ion (V_{ep}) is expressed as a function of the PEG concentration, [PEG], as described in our previous paper:¹⁵

$$V_{ep}/V_{ep,o} = V_{ep,f}/V_{ep,o} + K[(V_{ep,c} - V_{ep,f})/V_{ep,o}][PEG] \quad (1)$$

where $V_{ep,f}$ and $V_{ep,c}$ are the electrophoretic velocity of the free analyte ion and the analyte-PEG complex, respectively, and K is the complex formation constant of the analyte with PEG. $V_{ep,o}$ is the electrophoretic velocity of a reference compound with $K \approx 0$ (2CHO-BA in our case). In our experiments, $V_{ep}/V_{ep,o}$ exhibited linear relations against [PEG] up to 7.5 % (v/v) (data not shown, see Figure 2 in reference 15 as an example). Values of K can be easily estimated from the slopes of the linear plots on the basis of a reasonable assumption that $V_{ep,c} - V_{ep,f} \approx -V_{ep,f}$. Figure 2 shows the dependence of the K values of the analytes on the capillary temperature. In the cases of the four benzoic acids having a hydrogen-donating substituent (4CH₃CONH-BA, 4NH₂-BA, 4OH-BA, 2OH-BA), the K values decrease significantly with the raise in the capillary temperature. Contrastively, in the case of 4CHO-BA, 4CH₃-BA, 4CH₃COO-BA, and BA which have no hydrogen-donating active substituent, the K values are almost independent of the capillary temperature or the dependence is very small. These results support that the interaction between PEG and the four substituted benzoic acids with hydroxyl, amide, or amino group is predominantly governed by the hydrogen-bonding complex formation, in which the polyether oxygen atoms of PEG serve as hydrogen acceptors.

The above argument might mean that a raise in the capillary temperature is almost equivalent to a decrease in the PEG concentration. However, the two parameters controlling experimental conditions are not exactly identical with each other. Therefore, from a point of view of practical application, the capillary temperature as well as the PEG concentration can be a novel parameter to improve separations in this method. In our experiment, the best separation was achieved at 80 °C (Figure 1, B) for the samples used. Even when the use of a higher concentration of PEG is unavoidable to improve separation, a raise in the capillary

temperature would be occasionally useful for speedy separation, because it results in a decrease of the viscosity and then of the migration time.

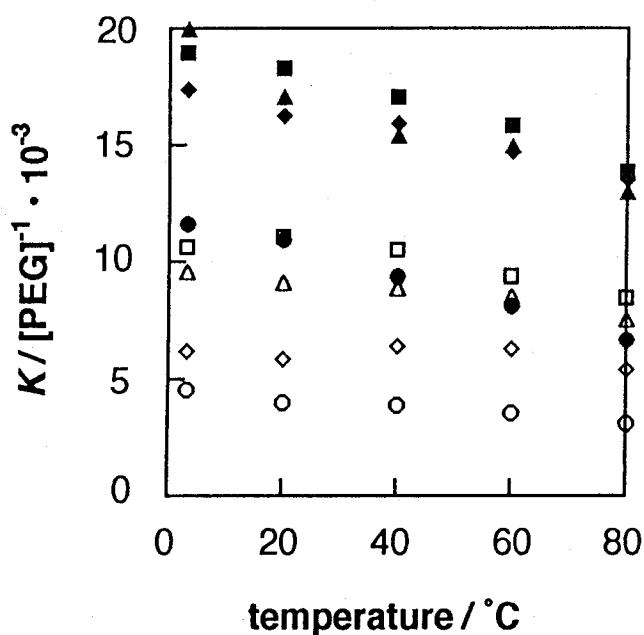


Figure 2 Dependence of the complex formation constant (K) on the capillary temperature:

(■) 4CH₃CONH-BA, (▲) 4OH-BA, (◆) 2OH-BA, (●) 4NH₂-BA, (□) 4CH₃-BA, (Δ) 4CH₃COO-BA, (◇) 4CHO-BA and (○) BA.

Urea Effect

Urea works as a bifunctional hydrogen donor and acceptor and it is often employed to break intra- and/or inter-molecular hydrogen-bonding of biological molecules, such as proteins and DNA. Considering that the hydrogen-bonding interaction works on the present separation mode for some analytes, urea to be added in the separation matrices is expected to compete with PEG (hydrogen acceptor) to form hydrogen-bonding complexes with the analytes (hydrogen donors) and/or with analytes to form hydrogen-bonding complexes with PEG. Our assumption here is that the electrophoretic velocity of the urea-analyte hydrogen-bonding complex is not far from that of the free analyte, because the molecular weight of urea is sufficiently small compared with that of PEG. This assumption leads into an expectation that an apparent value of K evaluated by eq 1 will decrease at a sufficiently high concentration of urea.

Table I. Effects of addition of 10 M urea on the K values

[Urea] / M	$K \times 10^3 / [\text{PEG}]^{-1,a}$	$K \times 10^3 / [\text{PEG}]^{-1,a}$
	0	10
4OH-BA	10.3	5.3
2OH-BA	9.8	5.6
4CH ₃ CONH-BA	9.5	3.9
4NH ₂ -BA	6.6	3.6
4CH ₃ -BA	7.0	3.6
4CH ₃ COO-BA	4.8	1.5
4CHO-BA	4.5	2.3
BA	2.7	2.3

[PEG] in percent (v/v).

The following experiments confirm the expectation. Urea was added at 10 M into an electrolyte solution of 10 mM phosphate buffer (pH 7.8) containing several concentrations of PEG. In this work, PEG 400 was used, because urea is almost insoluble in an aqueous solution of PEG 4000 at 5% (v/v). The apparent K value was evaluated based on eq 1. Plots of $V_{ep}/V_{ep,o}$ vs. [PEG] gave linear lines in the range of [PEG] from 2.5 to 10 % (v/v). Table I summarizes the K values of the samples in the absence and presence of 10 M urea. For all the analytes, the K values at 10 M urea decreased down to 30-85 % of those in the absence of urea. This decrease in K values is attributable to the suppression of the interaction, most significantly the hydrogen-bonding interaction, between PEG and the analytes. However, the urea effect was not restricted to the hydrogen-donating analytes. As will be mentioned later, we consider that there exist several minor interactions besides the relatively strong hydrogen-bonding interaction between PEG and benzoic acids. Therefore, urea seems to suppress those minor interactions also by the hydrogen-bonding complex formation with PEG molecules.

NMR Spectral Measurements

Nuclear magnetic resonance spectroscopy (NMR) is suitable for observing electrostatic interaction of molecules in solutions. When electrostatic interaction of an analyte with PEG occurs, the distribution of the electron density of the analyte should change, resulting in a change in the chemical shifts. In this study, the interaction of the phenolic hydroxyl group with PEG 20000 was followed in chloroform-D ($CDCl_3$) with 1H -NMR spectroscopy. Phenol was used as a model compound, because 4OH-BA is insoluble in $CDCl_3$.

Figure 3 (A) shows 1H -NMR spectra of 10 mM phenol in the absence and presence of PEG 20000 at concentrations of 1-5% (v/v). The spectral change upon the PEG addition was demonstrated by focusing our attention on the signals of the hydroxyl proton. The 1H signal of the hydroxyl group of phenol (4.8 ppm at 0% PEG) shifts significantly to a lower magnetic field on the addition of PEG. This provides a direct evidence for the hydrogen-bonding complex formation

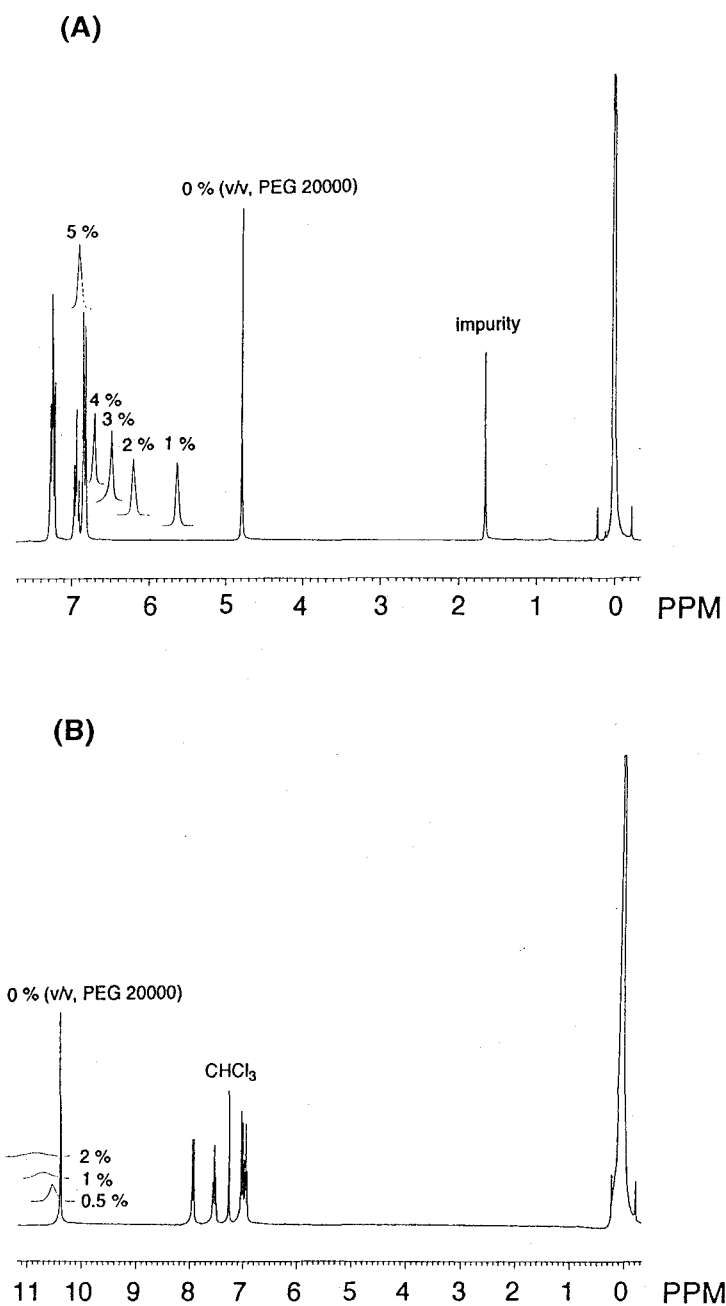


Figure 3 ^1H -NMR spectra of phenol (A) and 2OH-BA (B) in CDCl_3 with increasing concentration of PEG 20000.

between the phenolic hydroxyl group and the polyether oxygen atoms of PEG. The electrostatic attractive interaction (hydrogen-bonding complex formation) between the phenolic hydroxyl group and PEG should make the O-H bond length elongated. Thus the interaction decreases the electron density around the proton, resulting in the lower-field shift owing to the deshielding. Similar lower magnetic field shifts of phenolic and alcoholic ^1H signals are observed at increased concentrations as a result of intermolecular hydrogen bonding with themselves.¹⁷

PEG molecule has two hydroxyl groups at the ends. These end groups are reasonably considered to serve as hydrogen donors. This consideration can be supported by NMR experiments. The NMR signal of the end hydroxyl groups of PEG is broadened and shifted from 2.3 ppm to a lower magnetic field with addition of BA (data not shown). This indicates the possibility of the occurrence of the hydrogen-bonding interaction between the carboxylate group of BA and the end hydroxyl groups of PEG at least in CDCl_3 . The numbers of the hydroxyl groups in PEG molecules are generally far less than those of the ether oxygen atoms. Thus the hydrogen-donating property of the end groups of PEG would not so significant compared with the hydrogen-accepting property of the polyether segments. In the following, we will refer the hydrogen-bonding interaction derived from the polyether segments and the end hydroxyl groups as the major and minor hydrogen-bonding interactions, respectively.

Hydrophobic Interaction

Judging from the K values summarized in Table 1, $4\text{CH}_3\text{-BA}$ seems to interact attractively with PEG in a strength comparable to that of $4\text{NH}_2\text{-BA}$. This could not be simply explained in terms of the hydrogen-bonding interaction, because the methyl group is inactive in hydrogen bonding. PEG can afford more or less hydrophobic surroundings. This property seems to work as an another sub-mode in addition to the hydrogen-bonding mode for separation in the CE system using PEG. Such hydrophobic interaction with PEG could occur in the other analytes, but it was not be so remarkable as in the case of $4\text{CH}_3\text{-BA}$.

In addition to the direct effect of the hydrophobicity of PEG, the secondary effect might play a role in the hydrogen-bonding complex formation between analytes and PEG. That is a solvation effect. Hydrogen-bonding interaction with PEG will occur more effectively in hydrophobic surroundings than in aqueous phase. The benzoic acids used here are more or less hydrophobic in nature even in their mono-anion state. Thus the analytes will be surrounded by the hydrophobic segments (or hydrophobic pockets) of PEG in part. The hydrophobic environments will enhance the hydrogen-bonding interaction in this separation system.

Comparison between ortho- and para-Isomers

The K values of 4-acetoxybenzoic acid (4CH₃COO-BA) and 4CHO-BA were much larger than those of the *ortho*-isomers (2CH₃COO-BA and 2CHO-BA), respectively (K values of 2CH₃COO-BA were 1.4×10^{-3} [PEG 400]⁻¹ and 3.8×10^{-3} [PEG 4000]⁻¹)¹⁵. This seems to suggest steric hindrance against the hydrophobic interaction of the analytes with the polyether segments of PEG and/or the minor hydrogen-bonding interaction in part (Note here that the major hydrogen-bonding interaction is not expected for these analytes). This steric effect seems to be a minor one, but it is occasionally useful for separation. A typical example is the separation between 2CHO-BA and 4CHO-BA. The two analytes could not be separated in the absence of PEG in our experimental conditions because of their hydrodynamic radius close to each other. However, the addition of PEG 4000 (more than 2.5 % (v/v)) achieved the complete separation between them with the use of this steric effect. This would mean in turn that 2CHO-BA scarcely interacts with PEG. Thus 2CHO-BA is the best reference substance in the estimation of the complex formation constant of analytes with PEG (K) (see above).

Somewhat complicated situation is observed for 2OH-BA. Even though 2OH-BA is the *ortho*-isomer of 4OH-BA, the K values of 2OH-BA was comparable with that of 4OH-BA (Figure 2, Table 1). This result is interpretable as follows. The hydroxyl group of 2OH-BA as well as 4OH-BA is a strong hydrogen donor. Thus the major hydrogen-bonding interaction is reasonably

considered to govern the overall interaction with PEG predominantly and is sufficiently larger than the steric hindrance effect observed for other *ortho*-isomers. Another reason may be concerned with the hydrodynamic radius of 2OH-BA. In the absence of PEG, 2OH-BA migrates faster than 4OH-BA.¹⁵ This is in contrast with the case of 2CHO-BA and 4CHO-BA, which migrate simultaneously in the absence of PEG (see above). This result means that the hydrodynamic radius of 2OH-BA is smaller than that of 4OH-BA. Figure 3 (B) shows ¹H-NMR spectra of 10 mM 2OH-BA in the absence and presence of PEG 20000 at concentrations of 0.5-2% (v/v). The chemical shift of the hydroxyl proton of 2OH-BA is observed at 10.4 ppm. This low-field resonance indicates the intramolecular hydrogen-bonding formation between the hydroxyl group and the dissociated carboxyl group. Therefore, the small hydrodynamic radius compared with 4OH-BA can be attributable to the intramolecular hydrogen-bonding formation. With the addition of PEG, the ¹H signal of the hydroxyl group is broadened and disappeared. This phenomenon indicates the formation of the intermolecular hydrogen-bonding complex with PEG. Once the interaction with PEG occurs, the hydroxyl group bounded with the carboxylate group of 2OH-BA will turn out to form the complex with PEG. This will result in a larger increase in its apparent hydrodynamic radius than that expected for 4OH-BA. Such an amplified effect may cancel the steric hindrance effect observed for other *ortho*-isomers.

SUMMARY

Poly(ethylene glycol) (PEG) serves as a novel matrix in capillary electrophoresis. The purpose of this article is to explore some evidence for hydrogen-bonding complex formation between analytes and PEG in the separation system using benzoic acids as model analytes. A raise of the column temperature resulted in a significant decrease in the interaction between PEG and substituted benzoic acids with hydrogen-donating groups. Urea added as an additive suppressed the interaction. NMR spectra of phenol and salicylic acid in the presence of PEG in CDCl_3 proved obvious electrostatic interaction, most probably hydrogen-bonding interaction, between the hydroxyl protons of the analytes and PEG. These results strongly support that the hydrogen-bonding interaction between the polyether segments of PEG and the hydrogen-donating groups of analytes occurs in the separation systems. Some other minor interactions controlling the separation are also described.

REFERENCES

- (1) B. L. Karger, A. S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585.
- (2) G. M. McLaughlin, J. A. Nolan, J. L. Lindahl, R. M. Palmieri, K. W. Anderson, S. C. Morris, J. A. Morrison and T. J. Bronzert, *J. Liq. Chromatogr.*, 15 (1992) 961.
- (3) C. A. Monig and R. T. Kennedy,, *Anal. Chem.*, 66 (1994) 280R.
- (4) C. J. Pedersen, *J. Am. Chem. Soc.*, 89 (1967) 7017
- (5) C. J. Pedersen, *J. Am. Chem. Soc.*, 92 (1970) 386.
- (6) H. K. Frensdorff, *J. Am. Chem. Soc.*, 93 (1971) 600.
- (7) H. K. Frensdorff, *J. Am. Chem. Soc.*, 93 (1971) 4684.
- (8) T. Takeda and H. Goto, *Bull. Chem. Soc. Jpn.*, 52 (1979) 1920.
- (9) T. Takeda, *Bull. Chem. Soc. Jpn.*, 53 (1980) 2393.
- (10) T. Takeda and Y. Matsumoto, *Bull. Chem. Soc. Jpn.*, 60 (1987) 2313.
- (11) T. Takeda, Y. Kimura, Y. Kudo, Y.; Matsuda, H. *Bull. Chem. Soc. Jpn.*, 62 (1989) 2885.
- (12) Y. Kikuchi, N. Takahashi, T. Suzuki and K. Sawada, *Anal. Chim. Acta*, 256 (1992) 311.
- (13) L. F. Lindoy and G. W. Walker, *J. Am. Chem. Soc.*, 112 (1990) 3659.
- (14) P. S. K. Chia, L. F. Lindoy, G. W. Walker and G. W. Evertt, *J. Am. Chem. Soc.*, 113 (1991) 2533.
- (15) Y. Esaka, Y. Yamaguchi, K. Kano, M. Goto, H. Haraguchi and J. Takahashi, *Anal. Chem.*, 66 (1994) 2441.
- (16) Y. Esaka, Y. Yamaguchi, K. Kano and M. Goto, *J. Chromatogr. A* , 652 (1993) 225.
- (17) R. M. Silverstein, G. C. Bassler and T. C. Morrill, *Spectrometric Identification of Organic Compounds (fifth edition)*, Tokyo Kagaku Dohjin, Tokyo, 1992; p 170. (Authorized translation from English language edition published in 1991 by John Wiley & Sons, Inc., New York.)

1.3 Hydrogen-Bonding Interaction-Assisted Micellar Electrokinetic Chromatography Using Mixed Surfactant Systems

Micellar electrokinetic chromatography (MEKC) using ionic surfactant-micelles as pseudo-stationary phases and carriers has been developed as one of the separation modes in capillary electrophoresis and is applicable to the separation of neutral as well as charged analytes.^{1,2} The separation mechanism in MEKC is fundamentally based on the distribution of analytes between the pseudo and aqueous phases. A variety of ionic surfactants have been examined to realize novel selectivity in MEKC. Sodium dodecyl sulfate (SDS) is most frequently used in MEKC, in which the distribution is predominantly governed by the hydrophobic interaction between analytes and the hydrocarbon chain of SDS. Several “functional” moieties in other surfactant molecules can offer additional or cooperative interaction-fields. Chiral surfactants such as sodium *N*-dodecanol-*L*-valinate,³⁻⁶ *N*-dodecanol-*L*-glutamate,⁷ and various bile salts⁸⁻¹¹ have been utilized for chiral separation. Anionic surfactants having polar moieties besides the charged head group exhibit unique separation selectivity, which is clearly different from that of SDS.¹²

Such additional and/or specific interactions can be directly incorporated into MEKC mode by utilization of mixed surfactant systems. For example, the anionic chiral surfactants mentioned above are coupled together with “achiral” SDS to improve separation efficiency and also peak shapes.^{6,8} Mixed micelles composed of SDS and non-ionic chiral surfactants such as digitonin have been reported to provide “charged and chiral-selective” pseudo-stationary phases for chiral separation.^{5,7} Incorporation of sodium 1-octane sulfate (SOS) into the SDS micelle produces a novel mixed micelle for separation of catechols,¹³ in which SOS

is different from SDS simply in the hydrocarbon chain length.

In our previous papers,^{14,15} we have reported a hydrogen-bonding mode capillary zone electrophoresis (CZE) using poly(ethylene glycol) (PEG) as a matrix. The polyether oxygen atoms of PEG appear to serve as the hydrogen acceptors to form hydrogen-bonding complexes with analytes having hydrogen-donating activities during migration. The net strength of the interaction and then the separation characteristics can be controlled by the PEG concentration. The hydrogen-bonding interaction would be enhanced in hydrophobic circumstances such as micelle core compared with aqueous one. With these in mind, we have attempted to make the hydrogen-bonding interaction to assist the MEKC separation using a new type of mixed micelle composed of SDS and non-ionic surfactants, such as Brij 35 and Tween 20, having polyether moieties.

EXPERIMENTAL

Brij 35 (polyoxyethylene(23) laurylether), Tween 20 (polyoxyethylene-sorbitan monolaurate), and SDS were purchased from Wako Chemical (Tokyo, Japan), Kishida Chemical (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively, and used as received. Aniline ($\text{NH}_2\text{-Ph}$), phenol (HO-Ph), acetanilide ($\text{CH}_3\text{CONH-Ph}$), benzaldehyde (CHO-Ph), phenyl acetate ($\text{CH}_3\text{COO-Ph}$), *p*-toluidine ($4\text{NH}_2\text{-To}$), *p*-cresol (4HO-To), *p*-acetotoluidine ($4\text{CH}_3\text{CONH-To}$), *p*-tolualdehyde (4CHO-To), and *p*-cresol acetate ($4\text{CH}_3\text{COO-To}$) as model analytes were obtained from Wako Chemical, Tokyo Kasei Kogyo, or Nacalai Tesque. Oil yellow (Nacalai Tesque) was used as a marker of the micelle migration. All other chemicals were of analytical reagent grade.

Electrophoretic separation was performed using a Jasco CE-800 system (Tokyo, Japan) coupled to a Jasco 807-IT integrator. A capillary with 0.05-mm

i.d. and a column length of 500 mm was supplied from Jasco. In this column 300 mm was the effective length for separation. As electrolysis solutions, 10 mM phosphate buffer (pH 7.8) or 100 mM Tris-100 mM borate buffer (pH 8.2) was used. Prior to electrophoresis, the capillary was rinsed with 0.1 M sodium hydroxide for 10 min, water for 5 min, and then with the electrolysis solution used for separation for 5 min using an aspirator. Between the repeated analyses, the capillary was washed with the electrolysis solution for 5 min. Samples were introduced by siphoning at a height of 15 cm for a 5-10-s period. The detection of the analytes was performed by UV at a wavelength of 210 nm.

A series of ^1H NMR measurements of the mixed surfactant micellar systems were carried out in D_2O (Commissariat à l'Energie Atomique, Cedex, France) with JEOL GX-270 (Tokyo, Japan) operating at 270 MHz using 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (Nacalai Tesque) as a reference.

RESULTS AND DISCUSSION

Electropherograms in the mixed micellar EKC

Figure 1(A) shows an electropherogram of the ten mono- and di-substituted benzenes in the SDS-MEKC mode in the absence of non-ionic surfactant in phosphate buffer. The elution order essentially reflects the hydrophobicity of the analytes. Under the present conditions, the separation between $\text{NH}_2\text{-Ph}$ (peak 1) and HO-Ph (peak 2) and that among 4HO-To (peak 5), $\text{CH}_3\text{COO-Ph}$ (peak 6), and $4\text{NH}_2\text{-To}$ (peak 7) was not complete. Among these analytes separated incompletely, HO-Ph , 4HO-To , $\text{NH}_2\text{-Ph}$, and $4\text{NH}_2\text{-To}$ can inherently act as the donor in hydrogen bonding.

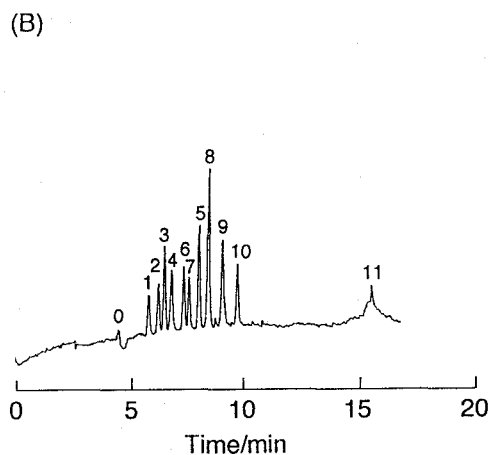
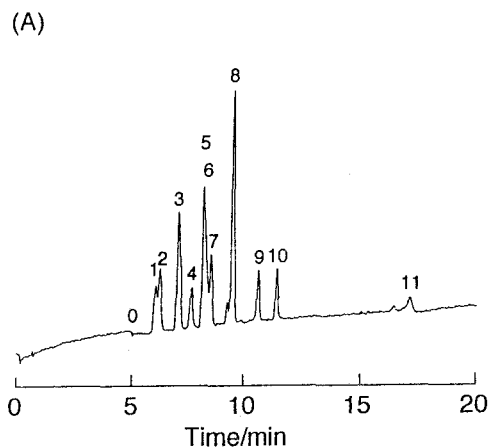


Figure 1 Micellar EKC separation of ten mono- or di-substituted benzenes in the absence (A) and presence of 3.5 mM Tween 20 (B) in electrolyte solution containing 50 mM SDS.

Peaks: (0) methanol (a marker of electroosmotic flow) (1) $\text{NH}_2\text{-Ph}$ (2) HO-Ph (3) $\text{CH}_3\text{CONH-Ph}$ (4) CHO-Ph (5) 4HO-To (6) $\text{CH}_3\text{COO-Ph}$ (7) $4\text{NH}_2\text{-To}$ (8) $4\text{CH}_3\text{CONH-To}$ (9) 4CHO-To (10) $4\text{CH}_3\text{COO-To}$ (11) oil yellow (a marker of micelle)

Based on our previous CZE experiments using polyethers,^{14,15} these hydrogen-donating analytes are expected to interact attractively with polyether segments. Thus, Tween 20 or Brij 35 was added at concentrations ranging from 1 to 50 mM to the electrolyte solution containing 50 mM of SDS, where the non-ionic surfactants and SDS are considered to form a mixed micelle (see later). The addition of Tween 20 provides significant change in the electrophoretic behavior. Figure 1(B) shows an electropherogram of the model sample analytes in the MEKC mode in the presence of 3.5 mM Tween 20 and 50 mM SDS. Under the present conditions, almost complete baseline separation was achieved. The most outstanding effect of the addition of Tween 20 is an relative increase in the migration time of the phenolic compounds (HO-Ph and 4HO-To) compared with those of the other analytes, resulting from the specific enhancement of the transfer of the phenolic compounds into the negatively charged mixed micelle. This is reasonably considered to occur through electrostatic interaction, most probably hydrogen-bonding interaction, between the hydroxyl groups of the two analytes and the polyether segments of the non-ionic surfactant in the mixed micelle.

Similar effect was observed using SDS/Brij 35 system in Tris-borate buffer and the reproducibility of the migration was superior to the SDS/Tween 20 system in phosphate buffer. However, the use of Tris-buffer for the SDS/Brij 35 system resulted in a decline of the peak resolution even in the absence of Brij 35. In phosphate buffer the SDS/Brij 35 system was not acceptable most probably due to the adsorption of Brij 35 on the inner wall of the capillary.

Thermodynamic aspect of the mixed micellar EKC

Here we try quantify the energetic effects of the addition of the non-ionic surfactant on the migration behavior. In MEKC, solutes (analytes) are reasonably considered to be dissolved in equilibrium between aqueous (Aq) and micellar

phases (M). The chemical potential of the analyte in the aqueous phase (μ_{Aq}) is expressed as

$$\mu_{Aq} = \mu^{\circ}_{Aq} + RT \ln X_{Aq} \quad (1)$$

where X_{Aq} is the concentration of the solute in mol fraction units and μ°_{Aq} the standard chemical potential on the unitary scale.¹⁶ Assuming that the solute within the micelle constitutes an ideal solution, we can use the analogue of eq 1 for the micellar phase

$$\mu_M = \mu^{\circ}_M + RT \ln X_M \quad (2)$$

Thus, the standard free energy of the transfer of the solute to the micellar interior from aqueous phase (that is, the difference between the free energy of the interaction with the solvents; $\Delta\mu^{\circ} \equiv \mu^{\circ}_M - \mu^{\circ}_{Aq}$) is given by

$$\Delta\mu^{\circ} = -RT \ln(X_M / X_{Aq}) \quad (3)$$

Under the conditions of that X_M and $X_{Aq} \ll 1$, X_M / X_{Aq} can be related to the distribution constant (K) of the analyte by

$$X_M / X_A \approx K(C_{water,Aq} / C_{sf,M}) \quad (4)$$

where $C_{water,Aq}$ and $C_{sf,M}$ are the mol concentrations of water in aqueous phase and of surfactant in micelles, respectively. At low micellar concentrations, the capacity factor (k') in MEKC can be expressed by

$$k' \approx K\nu_M(C_{sf} - C_{cmc}) \quad (5)$$

where ν_M , C_{sf} , and C_{cmc} represent, respectively, the partial specific volume of the micelle, the analytical concentration of the surfactant, and the critical micellization concentration (CMC) of the surfactant system.² Combining eqs 3-5, the transfer free energy can be related to k' as follows

$$\Delta\mu^{\circ} = -RT \ln(k' \Phi) \quad (6)$$

where $\Phi [= (C_{\text{water,Aq}}/C_{\text{sf,M}})/\nu_{\text{M}}(C_{\text{sf}} - C_{\text{cmc}})]$ is a parameter characteristic of the micellar system alone and *independent of solutes*.

The transfer free energy of (neutral) solutes in the pure SDS micellar system ($\Delta\mu^\circ$ in eq 6) is predominantly ascribed to the hydrophobic interaction between the analytes and the hydrophobic alkyl chain of SDS and then it is denoted by $\Delta\mu^\circ_{\text{HP}}$. On the other hand, we propose a thermodynamic model of our mixed micellar system consisting of SDS and non-ionic surfactant as follows. The hydrogen-bonding interaction is considered to work additively against the hydrophobic interaction. At low mixing ratios of non-ionic surfactant, we assume that the hydrophobic interaction is practically independent of the analytical concentration of the non-ionic surfactant (C_{n}) and that the additional stabilization energy caused by the incorporation of the non-ionic surfactant is proportional to the concentration of the non-ionic surfactant molecules in the mixed micelle ($C_{\text{n,M}}$). Therefore, the transfer free energy in the mixed micellar system ($\Delta\mu^\circ_{\text{MM}}$) can be expressed by

$$\Delta\mu^\circ_{\text{MM}} = \Delta\mu^\circ_{\text{HP}} - kfC_{\text{n}} \quad (7)$$

where k is a constant representing the stabilization energy per unit concentration of the non-ionic surfactants in the mixed micelle and f is a condensation factor given by $f = C_{\text{n,M}}/C_{\text{n}}$. Substitution of $\Delta\mu^\circ_{\text{MM}}$ in eq 7 into $\Delta\mu^\circ$ of eq 6 yields the following equation describing the relation between k' and C_{ns} .

$$RT \ln k' = kfC_{\text{ns}} - \Delta\mu^\circ_{\text{HP}} - RT \ln \Phi \quad (8)$$

Here, Φ in this equation is also a function of C_{n} . In order to eliminate the C_{n} dependence of Φ , we will offer a relative value of k' of a given analyte against that of a certain reference compound (k'_0), since Φ is independent of solutes as described above. Thus, we can get

$$RT \ln(k'/k'_o) = (k - k_o) f C_n - (\Delta\mu^\circ_{HP} - \Delta\mu^\circ_{HP,o}) \quad (9)$$

where k'_o and $\Delta\mu^\circ_{HP,o}$ denote the corresponding values of the reference compound.

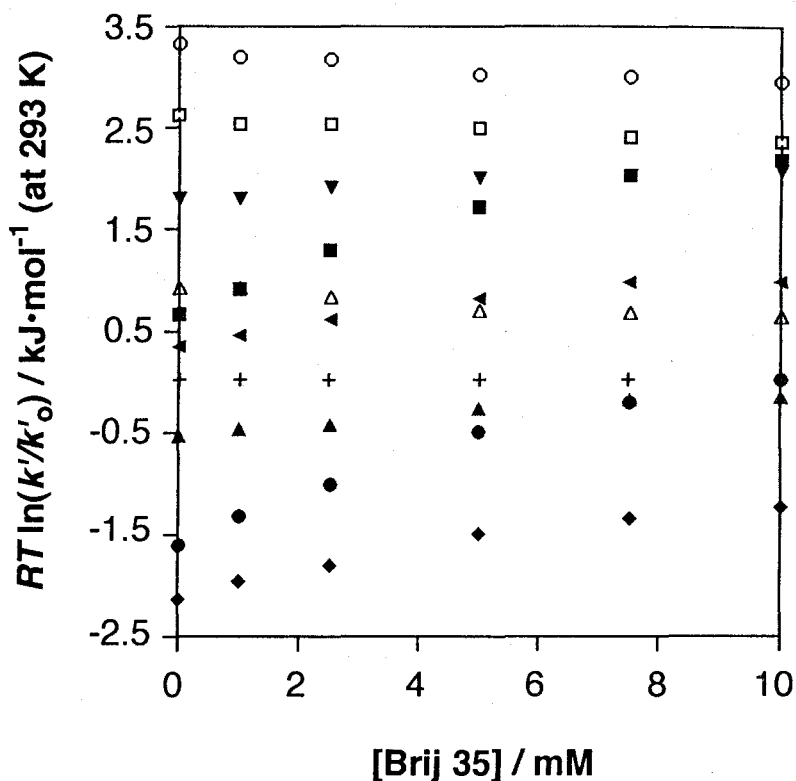


Figure 2 Dependence of $RT \ln(k'/k'_o)$ of the substituted benzenes on the concentration of Brij 35.

Symbols: (●) HO-Ph (■) 4HO-To (◆) NH₂-Ph (◄) 4NH₂-To (▲) CH₃CONH-Ph (▼) 4CH₃CONH-To (Δ) CH₃COO-Ph (○) 4CH₃COO-To (◻) 4CHO-To (+) CHO-Ph (as a reference compound)

Figure 2 shows the dependence of $RT \ln(k'/k'_o)$ on the analytical concentration of Brij 35 ([Brij 35]), where CHO-Ph was selected as a reference compound because the interaction of CHO group with PEG appears to be negligible in CZE mode.^{14,15} The capacity factors were evaluated according to the relation

$$k' = (t_R - t_o) / \{t_o(1 - t_R/t_{mc})\} \quad (10)$$

where t_R , t_o , and t_{mc} are the migration time of the analyte, water, and the micelle, respectively.^{1,2} The values of $RT \ln(k'/k'_o)$ show linear relationships against [Brij 35] at [Brij 35] < 5 mM. The linear relationship is well described by eq 9. Thus the slope gives the $(k - k_o)f$ value which reflects a relative magnitude of the additional interaction with Brij 35 compared with CHO-Ph, while the intercept represents the difference in the hydrophobic stabilization energy in the pure SDS micellar system between a given analyte and CHO-Ph ($\Delta\mu^{\circ}_{HP,o} - \Delta\mu^{\circ}_{HP}$). Table 1 summarizes the $(k - k_o)f$ values thus evaluated for the ten substituted benzenes. The phenolic compounds (HO-Ph and 4HO-To) with strong hydrogen-donating activity yielded the largest values of $(k - k_o)f$ over 200 kJ mol⁻¹ M⁻¹, indicating the strong attractive interaction with Brij 35. The other hydrogen-donating active analytes with the amino or amide group, (NH₂-Ph, 4NH₂-To, CH₃CONH-Ph, 4CH₃CONH-To) also gave positive values of $(k - k_o)f$. In contrast, $(k - k_o)f$ values of the hydrogen-donating inactive analyte (CH₃COO-Ph, 4CH₃COO-To, and 4CHO-To) were negative. These results are fundamentally described in terms of the hydrogen-bonding interaction. The best separation was achieved at [Brij 35] = 2 mM as judged from Fig. 2. In the case of the SDS/Tween 20 system also, similar relations between $RT \ln(k'/k'_o)$ and [Tween 20] were obtained, in which the best resolution was observed at [Tween 20] = 3.5 mM (Fig. 1).

Table 1. Slopes of $RT \ln(k'/k'_O)$ vs. [Brij 35] plots at 293 K

	Slope / $\text{kJ} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$
HO-Ph (phenol)	218
4HO-To (<i>p</i> -cresol)	210
NH ₂ -Ph (aniline)	124
4NH ₂ -To (<i>p</i> -Toluidine)	93
CH ₃ CONH-Ph (acetanilide)	52
4CH ₃ CONH-To (<i>p</i> -acetotoluidine)	42
CHO-Ph (benzaldehyde)	0
4CHO-To (<i>p</i> -tolualdehyde)	-24
CH ₃ COO-Ph (phenyl acetate)	-47
4CH ₃ COO-To (<i>p</i> -cresol acetate)	-54

Now let us consider the energetic contribution of the methyl substituent on the hydrophobic and hydrogen-bonding interactions. The intercepts in Fig. 2 (at [Brij 35] = 0) of the toluene derivatives (HO-To, NH₂-To, CH₃CONH-To, CHO-To, CH₃COO-To) are larger than those of the corresponding mono-substituted benzenes (HO-Ph, NH₂-Ph, CH₃CONH-Ph, CHO-Ph, CH₃COO-Ph) by about 2.4 kJ mol^{-1} , which corresponds to the hydrophobic stabilization energy associated with the transfer of the methyl group from aqueous to the SDS micelle ($-\Delta\mu^\circ_{\text{HP,Me}}$). This stabilization energy is rather smaller than that of the methyl group of linear hydrocarbons in SDS micellar system (ca. 4 kJ mol^{-1}).¹⁶ The discrepancy might be ascribed to the difference in the circumstances between the methyl groups attached to the aromatic carbon and the linear aliphatic carbon.

On the other hand, the slopes in Fig. 2 (*i.e.* the $(k - k_0)f$ values in Table 1) of the toluene derivatives (HO-To, NH₂-To, CH₃CONH-To, CHO-To, and CH₃COO-To) are almost the same as those of the corresponding mono-substituted benzenes (HO-Ph, NH₂-Ph, CH₃CONH-Ph, CHO-Ph, and CH₃COO-Ph). This result indicates that the methyl group which is incapable of hydrogen-bonding complex formation does not participate in the specific interaction with Brij 35 and also that the hydrophobic interaction remains almost unchanged during the addition of Brij 35. The two matters are in agreement with our thermodynamic model described above.

The energetic effect of the hydrogen-bonding interaction between the phenols and Brij 35 may be estimated as follows. The volume ratio of the aqueous and pseudo-stationary phases in our SDS-MEKC ($= [\nu_M(C_{sf} - C_{cmc})]^{-1}$ in eq 5) can be evaluated as 140 using $k' = 0.522$ for HO-Ph at [SDS]=50 mM and $K = 73$ for HO-Ph in the pure SDS micellar system.² Assuming that all of Brij 35 molecules are incorporated into the mixed micelle (that is, $f \approx 140$), values of $(k - k_0)$ of the phenols are evaluated as about $1.5 \text{ kJ mol}^{-1} \text{ M}^{-1}$. For imaginary pure solution of Brij 35, the mol concentration might be about 0.8 M (MW = 1214, $d \approx 1$). Therefore, the hydrogen-bonding interaction energy between the phenols and Brij 35 would be roughly estimated as about 1.2 kJ mol^{-1} . The energy thus evaluated seems to be smaller than that of the usual hydrogen-bonding. This would be mainly due to the weak hydrogen-accepting ability of Brij 35. However, the energy is enough to improve the selectivity in MEKC as described above.

All the behavior described above appear to be fundamentally in accord with our thermodynamic model. However, one of disaccording points is that the $(k - k_0)f$ values of NH₂-Ph and 4NH₂-To are larger than those of CH₃CONH-Ph

and 4CH₃CONH-To, in spite of that the amide group is stronger hydrogen donor than the amino group. Similar disaccordance is observed in the comparison of the $(k - k_0)f$ values between CH₃COO-Ph and CHO-Ph, or between 4CH₃COO-To and 4CHO-Ph: both acetoxy and formyl groups do not act as hydrogen-donors, but the $(k - k_0)f$ values of CH₃COO-Ph and 4CH₃COO-To are more negative than those of CHO-Ph and 4CHO-Ph. These phenomena are in contrast with those in CZE mode using PEG as matrix, in which the order of the interaction of the substituents with PEG is found as follows; OH > CONH > NH₂ >> CH₃COO ≥ CHO.^{14,15} The cause of the disaccordance is not clear. Some another interaction might work in this mixed micellar EKC mode.

Formation of mixed micelles

The CMC values of non-ionic surfactants are generally much lower than those of ionic surfactants as long as they have the same length of alkyl chain. Therefore almost all molecule of Tween 20 and Brij 35 would be considered to form mixed micelles with SDS molecules. The mixed micelle formation can be supported by the following two experimental results. The first concerns the EKC behavior. The k' values of the analytes with the strong hydrogen-donating activity, such as HO-Ph and 4HO-To, increased selectively and remarkably with an increase in [Tween 20] or [Brij 35] up to about 15 mM under our experimental conditions. Considering the relatively strong attractive interaction between these analytes and the polyether moieties of Tween 20 or Brij 35, the non-ionic surfactants-containing micelles which interact with the analytes should have negative charges in order to reduce the migration time of the analytes (Note here that the detection was performed at the negative potential side). This obviously indicates the formation of mixed micelles with SDS. The second is NMR spectroscopic evidence. The ¹H signal on the C1 and the C2 carbon atoms of SDS

shift to a slightly higher magnetic field with increasing [Tween 20] or [Brij 35] in the mixed surfactant systems. These shifts were ca. -0.020 ppm. for ^1H on C1 and ca. -0.015 ppm. for ^1H signal on C2 at [SDS] = 50 mM and [Brij 35] or [Tween 20] = 10 mM. This means the change in the electrostatic environment near the sulfonic group of SDS, most probably owing to the interaction of the sulfonic group with the polyether moieties of the non-ionic surfactants. Since the concentration of free Tween 20 or Brij 35 should be very low as described above, the ^1H signal shifts are reasonably ascribed to the formation of mixed micelles. In contrast, the ^1H signal on the C12 carbon atom (the methyl group) of SDS remained almost unchanged. This may support that the hydrophobic environment in the micellar core is scarcely affected by the addition of the non-ionic surfactants.

It is important to point out here that the addition of the non-ionic surfactants provide a significant change in the electrophoretic velocity of the mixed micelle ($V_{\text{ep,mc}}$). In this study, values of $V_{\text{ep,mc}}$ were estimated on the basis of their relationship with the net (measured) migration velocity of micelle (V_{mc}):

$$V_{\text{mc}} = V_{\text{ep,mc}} + V_{\text{eo}} \quad (11)$$

where V_{eo} is the electroosmotic velocity with the sign (plus) opposite to that of $V_{\text{ep,mc}}$ for the negatively charged micelles. The value of V_{eo} was evaluated from the migration time of methanol, which was co-injected with the samples. Figure 3 shows $V_{\text{ep,mc}}$ values as a function of [Brij 35] or [Tween 20]. The absolute values of $V_{\text{ep,mc}}$ decrease with increasing [Brij 35] or [Tween 20] in the concentration range from 1 to 15 mM. This seems to suggest an increase in the hydrodynamic radius of the micelles with the addition of the non-ionic surfactants.

At the concentrations over 15 mM of [Brij 35] or [Tween 20], however, $V_{\text{ep,mc}}$ became independent of [Brij 35] or [Tween 20] (Fig. 3). Under these

conditions, the capacity factor (k') of all analytes examined decreased remarkably with increase in [Brij 35] or [Tween 20] (data not shown). These phenomena suggest the occurrence of drastic change in structure of the mixed micelle. Anyway such serious decrease in k' values of all analytes results in poor separation of analytes. Thus, the practically available concentration of Brij 35 and Tween 20 would be less than 15 mM at least at [SDS] = 50 mM. Interestingly, such phenomena were not observed when Brij 30 was used instead of Brij 35 in the concentration range from 1 to 100 mM. Brij 30 has only four ethylene glycol segments, while 23 segments Brij 35 has. Therefore, relatively long polyether chains of Brij 35 or Tween 20 appear to be responsible for the occurrence of the drastic change in the structure of the mixed micelles. Detailed study for these phenomena is underway.

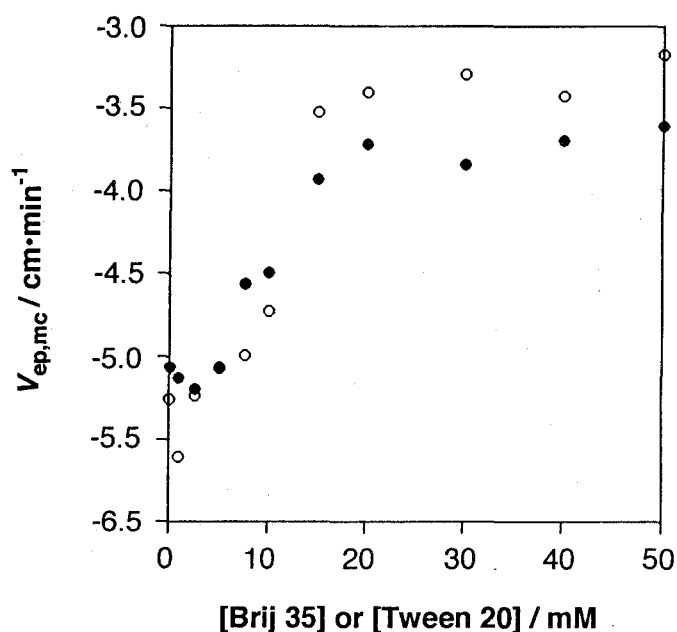


Figure 3 Electrophoretic velocity of the mixed micelles as a function of the concentration of Brij 35 (O) or Tween 20 (●).

SUMMARY

Micellar electrokinetic chromatography (MEKC) has been examined using mixed surfactant systems consisting of Brij 35 or Tween 20, non-ionic surfactants with the polyether structure, together with sodium dodecyl sulfate (SDS). Addition of the non-ionic surfactant into SDS micellar system provides selective increase in the relative capacity factors of some substituted benzenes having hydrogen-donating substituents such as hydroxyl, amino, and amide group. This effect can be ascribed to the hydrogen-bonding formation between these solutes and the polyether segments of the non-ionic surfactant. The hydrogen-bonding interaction appears to work additively against the hydrophobic interaction. The separation selectively can be well controlled by the mixing ratio of two surfactants. The thermodynamic aspect of the mixed micellar systems are discussed in detail.

REFERENCES

- (1) S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
- (2) S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985), 834.
- (3) A. Dobashi, T. Ono, S. Hara, J. Yamaguchi, *Anal. Chem.*, 61 (1989) 1984.
- (4) A. Dobashi, T. Ono, S. Hara, J. Yamaguchi, *J. Chromatogr.*, 480 (1989) 413.
- (5) K. Otsuka and S. Terabe, *J. Chromatogr.*, 515 (1990) 221.
- (6) K. Otsuka, J. Kawahara, K. Tatekawa and S. Terabe, *J. Chromatogr.*, 559 (1991) 209.
- (7) K. Otsuka, M. Kashihara, Y. Kawaguchi, R. Koike, T. Hisamitsu and S. Terabe, *J. Chromatogr. A*, 652 (1993) 253.
- (8) S. Terabe, M. Shibata and Y. Miyashita, *J. Chromatogr.*, 480 (1989) 403.
- (9) H. Nishi, T. Fukuyama, M. Masuo and S. Terabe., *J. Microcol. Sep.*, 1 (1989) 234.
- (10) H. Nishi, T. Fukuyama, M. Masuo, S. Terabe., *J. Chromatogr.*, 515 (1990) 233
- (11) R. O. Cole, M. J. Spaniak and W. L. Hinze, *J. High Resolut. Chromatogr.*, 13 (1990) 579.
- (12) M. Tanaka, T. Ishida, T. Araki, A. Masuyama, Y. Nakatsuji, M. Okahara and S. Terabe, *J. Chromatogr.*, 648 (1993) 469.
- (13) R. A. Wallingford, P. D. Curry and A. G. Ewing, *J. Microcol. Sep.*, 1 (1989) 23.
- (14) Y. Esaka, Y. Yamaguchi, M. Goto, H. Haraguchi, J. Takahashi and K. Kano, *Anal. Chem.*, 66 (1994) 2441.
- (15) Y. Esaka, M. Goto, H. Haraguchi, T. Ikeda and K. Kano, *J. Chromatogr. A*, in press.
- (16) C. Tanford, "The Hydrophobic Effect - Formation of Micelles and Biological Membranes" 2nd Ed., Wiley, New York, 1980, Chaps. 2,3, and 6.

CHAPTER 2

SEPARATION CHARACTERISTICS OF WHALE MYOGLOBIN BY CHROMATOFOCUSING

In recent years, trace elements in biology have been received great attention. Such studies have been promoted by the development of analytical techniques for trace metals such as atomic absorption spectrometry, and inductively coupled plasma mass spectrometry ¹. On the other hand, biological compounds including some specific metals have been found as metalloproteins, metalloenzymes and other metal-containing compounds. Usually metalloproteins and metalloenzymes contain some specific metals and provide specific biological or physiological functions. Although such great advances in the studies on metalloproteins and metalloenzymes have been achieved, efficient and specific separation of biological samples is still often difficult and is desirable to be developed. Hence in the present study CF which provides the information about isoelectric point (pI) ^{2,3} has been examined by using perm whale myoglobin containing iron in the active sites in order to evaluate the separation efficiency. A photodiode array detector was used to identify the separated compounds spectroscopically.

EXPERIMENTAL

Materials

Sperm myoglobin (M0380) as obtained from Sigma (USA) and used without further purification. Myoglobin (MBW-1) from Biozyme (USA) was also used for comparison. The pI marker set (pI Cariblation Kit Electran, range 5.65-8.3) was purchased from BDH Co. (USA). As the pI markers the following proteins were used (the numbering of the proteins are corresponding to the numbers of the peaks shown in the chromatogram at the lower part of Fig. 3; 1) azurin (pI = 5.65), 2) trifluoroacetylated myoglobin met (porcine, 5.92), 3) myoglobin (porcine, 6.45), 4) trifluoroacetylated myoglobin met (equine, 6.86), 5) myoglobin (equine, 7.30), 6) trifluoroacetylated myoglobin met (sperm whale, 7.65, 7.62), 7) myoglobin (sperm whale, 8.3). Ampholine carrier ampholytes solution of the pH

range 8-9.5 (LKB) was used as the mobile phase (elution buffer) in CF. All other chemicals used were of analytical reagent grade or LC grade.

Apparatus

A high performance liquid chromatography (HPLC) system, FPLC® system, used consisted of 2 pumps (P-500 from Pharmacia, Sweden), a LC controller LCC-500 PLUS (Pharmacia), an injector MV-7 (Pharmacia), UV-visible detector (2141 variable wavelength monitor from LKB, Sweden), and a chart recorder REC-482 (Pharmacia). A photodiode array detector SPD-M6A (Shimadzu, Japan) was operated with a personal computer PC-9801RA (NEC, Japan) and a printer PC-PR201CL (NEC) for the on-line measurement of the UV-visible absorption spectra of myoglobin in the eluent solutions. As the separation columns, Mono-P (200 mm × 5 mm i.d.) for CF and Superose 12 (300 mm × 10 mm i.d.) for SEC were employed, both of which were purchased from Pharmacia. A pH meter HM-208 (TOA, Japan) was used with a pH microelectrode GS-5016S (TOA) for the pH measurement.

The metal contents of peak components were determined by a AA 540-13 atomic absorption spectrometer (Shimadzu) with a graphite furnace atomizer.

RESULTS AND DISCUSSION

Separation of sperm whale myoglobin by chromatofocusing (CF) and size exclusion chromatography (SEC)

Sperm whale myoglobin was separated by CF, in which a 20 mM glycine-ammonia solution of pH 9.6 and a 0.2 % Ampholine -acetic acid solution of pH 7.8 were used as starting and eluting buffers respectively. The chromatogram of obtained is reproduced in Fig. 1 (a) which shows one large main peak and 8 small peaks. The ratio of the area of main peak to the sum of the areas of the small peaks was about 77:33.

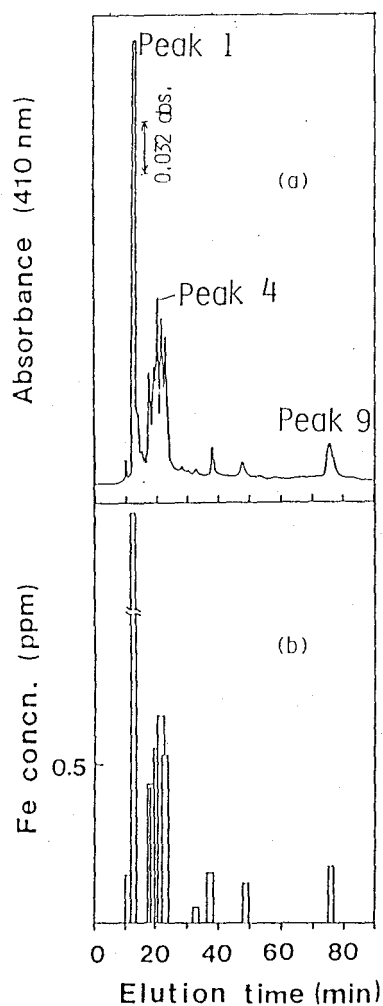


Figure 1 CF chromatograms of sperm whale myoglobin obtained by (a) UV detection and (b) iron determination.

Conditions: (for CF) Starting buffer, 20 mM glycine-NH₃ solution (pH 9.6); eluting buffer, 0.2% Ampholine-acetic acid solution (pH 7.8); flow rate, 1.0 ml/min; detection, 405 nm. (for AAS, see below.) $\lambda=248.3$ nm, drying; 150 °C, 40 sec, ashing; 750 °C, 30 sec, atomizing; 2000 °C, 6 sec.

In Fig. 2, SEC chromatograms for 9 peak fractions separated by CF are shown, where the 0.1 M phosphate buffer solution (0.1 M Na_2HPO_4 + 0.1 M KH_2PO_4) was used as the mobile phase with the flow rate of 0.5 ml/min. And the injection volume of the fraction was 200 μl . As can be seen in the figure, all fractions provide the response peaks at the same elution time in the chromatograms. This indicates that the components separated by CF have the same molecular weight, though they have different pI values, and they may not be the impurities.

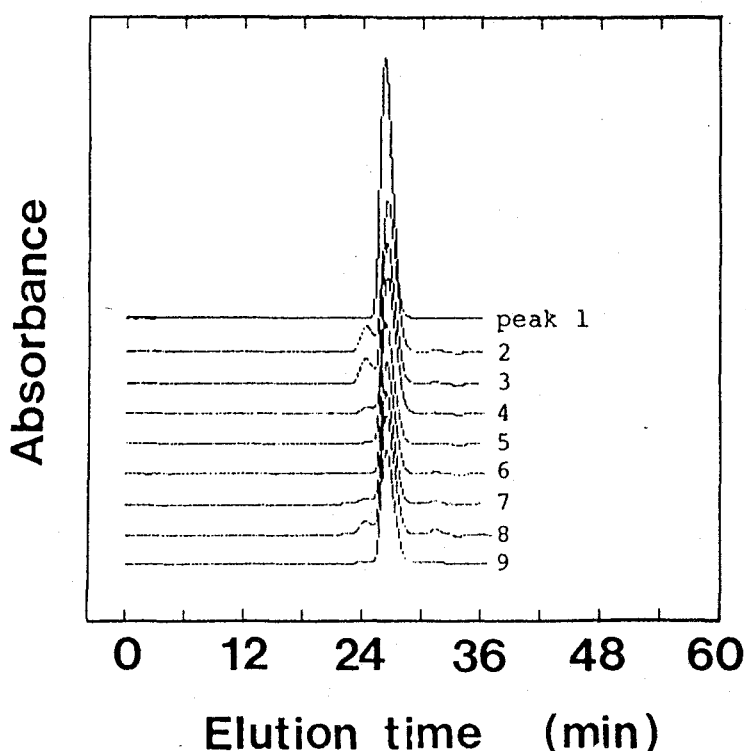


Figure 2 SEC chromatograms of the fractions containing 9 components separated by CF when myoglobin was injected

Eluent, 0.1 M phosphate buffer solution (0.1 M Na_2HPO_4 + 0.1 M KH_2PO_4); flow rate, 0.5 ml / min; detection, 405 nm

Determination of iron contents of the components separated by CF

The eluent of CF (Fig. 1 (a)) was fractionated to determine iron content by atomic absorption spectrometry (AAS) and the result is shown in Fig. 1 (b). As can be seen in Fig. 1 (a) and (b), the shape of the chromatogram obtained by determining the iron content of the fractions is almost identical with that obtained from UV detection. It indicates that all components in the fractions corresponding to the 9 peaks by CF have iron with a almost same content. In measurements of iron contents, the experimental conditions were as follows: $\lambda=248.3$ nm, drying; 150 °C, 40 sec, ashing; 750 °C, 30 sec, atomizing; 2000 °C, 6 sec.

The UV-vis spectra of the components separated by CF

In order to confirm the chemical forms in the peak fractions separated by CF, the UV-visible absorption spectra of the components in the fractions were measured by using a photodiode array detector. Some of the spectra corresponding to the peaks 1, 4 and 9 are shown in Fig. 3. In the measurement of the absorption spectra, the photodiode array detector was connected to the HPLC column directly. Furthermore, each spectrum was measured by injecting each fraction separated by CF into SEC column because some constituents in the carrier-ampholytes interfered with the spectral measurement, i.e., interfering constituents in the carrier ampholytes were separated by SEC from the myoglobin components in the fractions. All the spectra provided the absorption maxima near 410 nm and small peaks near 280 nm. These experimental results strongly suggest that all the components in the fractions separated by CF are myoglobin itself with different pI values.

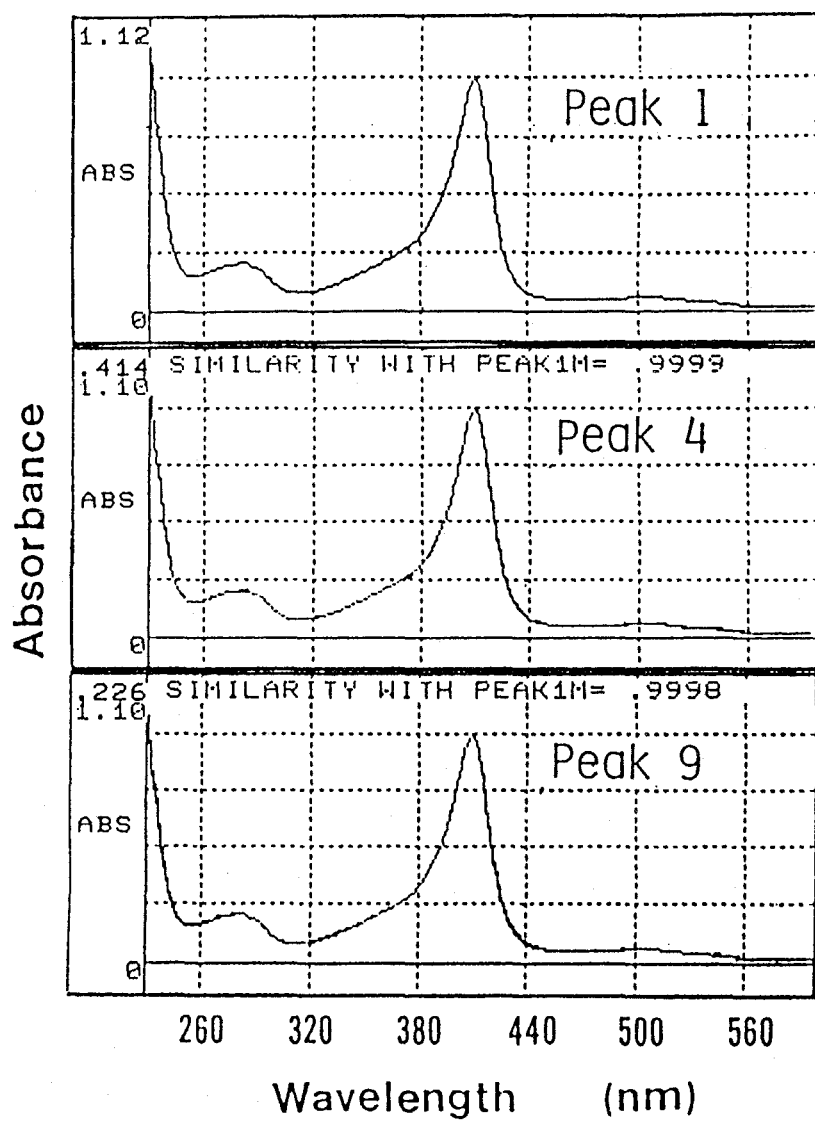


Figure 3 UV-visible absorption spectra of the components for peaks 1,4, and 9 which were separated by CF (Fig. 1 (a)).

Estimation of isoelectric points of the components separated by CF

In Fig. 4, the CF chromatogram for a mixture of proteins with different pI values is shown along with the pH variation curve of the CF eluent. As the initial buffer solution in CF, 25 mM triethylamine solution was used, where the pH of these solutions was adjusted to 10.6 with hydrochloric acid. The pH of the 0.2 % Ampholine carrier ampholytes solution as the eluent buffer solution was adjusted to 5.95 with hydrochloric acid solution. In this experiment, the pI markers which are commercially available as a pI marker set (pI Calibration Kit from BDH Co.) were used to calibrate the pH value of the CF eluent. The response peaks of the proteins examined were detected at 410 nm and 280 nm. The pH values of the eluent were measured at the column exit by using a pH microelectrode. It should be stressed here that the pI markers used in the present experiment have similar protein structures, i.e., all the proteins except for azurin are the myoglobins from different sources or its derivatives. This is very important to make the calibration curves for pI measurement because the correlation between the pI values of the proteins and pH values of the CF eluent was not so good under the present experimental conditions when the different types of proteins were used as the pI markers. As can be seen in Fig. 6-4, the proteins with the larger pI values are eluted at the higher pH and all the proteins are well separated by CF. Therefore, the pI calibration curve can be made as the elution time of the protein vs. the pH of the eluent. Using the pI calibration curve, the pI values of the 9 components for myoglobin separated by CF were estimated, and the results are summarized in Table 1. It is noted here that several isoforms of myoglobin with different pI values exist in sperm whale myoglobin, as can be seen in Table 1. At present, chemical or structural differences of such isoforms can not be elucidated, but the present experimental results suggest the heterogeneity of myoglobin as the protein. It should be added here that all the myoglobin isoforms of sperm whale myoglobin separated by CF were met-myoglobin.

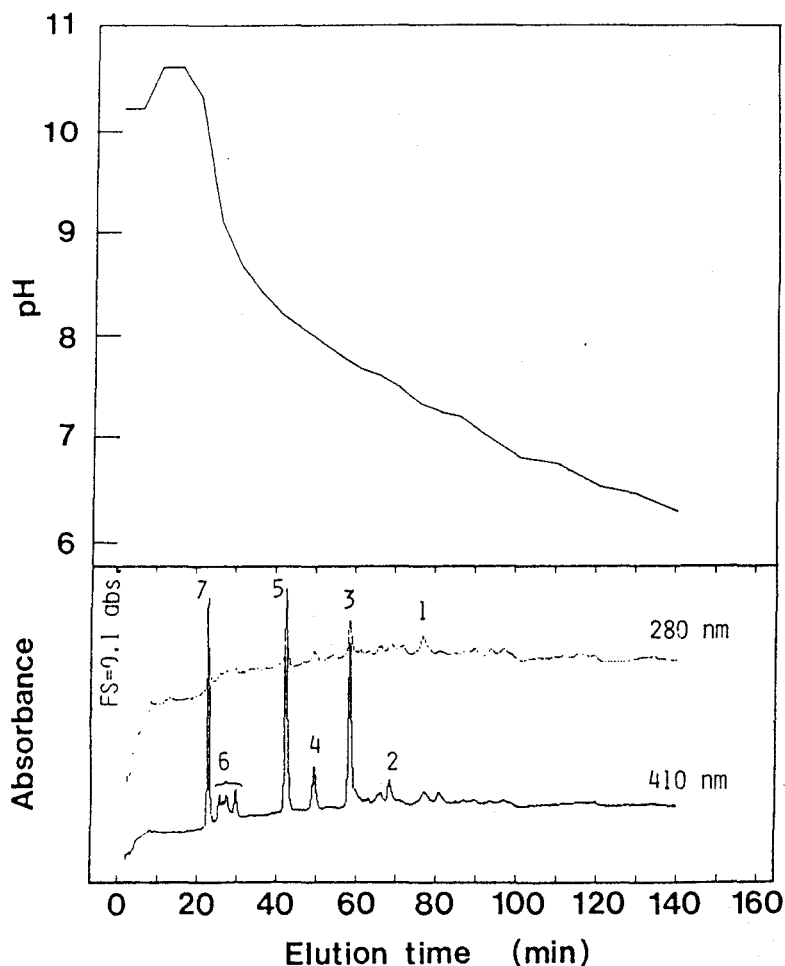


Figure 4 CF chromatogram of pI markers detected at 410 and 280 nm (lower) and the pH variation of the CF eluent (upper).

Starting buffer, 25 mM triethylamine-HCl solution, pH 10.6; eluting buffer, 0.2% Ampholine-HCl, pH 5.95; flow rate, 1.0 ml / min; Peaks, 1. azurin ($pI = 5.65$), 2. trifluoroacetylated myoglobin met (porcine, 5.92), 3. myoglobin (porcine, 6.45), 4. trifluoroacetylated myoglobin met (equine, 6.86), 5. myoglobin (equine, 7.30), 6. trifluoroacetylated myoglobin met (sperm whale, 7.65, 7.62), 7. myoglobin (sperm whale, 8.3).

Table 1 Estimated pI values of myoglobin components separated by CF

Peak number (in Fig. 1 (a))	pH of eluent	pI estimated
1	9.40	8.3
2	8.96	8.0
3	8.84	7.9
4	8.76	7.8
5	8.65	7.8
6	8.60	7.6
7	8.26	7.4
8	8.15	7.3
9	7.95	7.1

SUMMARY

Separation of sperm whale myoglobin (Mb) was studied by chromatofocusing (CF) and size exclusion chromatography (SEC). At least 9 peaks were observed in the CF chromatogram. This suggests the existence of 9 different components with different isoelectric points. On the other hand, all these components gave the same elution time with each other in SEC, when each peak fraction separated by CF was injected. Thus the separation characteristics in CF was investigated with aid of a photodiode array detector to elucidate the separated components.

REFERENCES

- (1) H. Haraguchi, *Inductively Coupled Plasma Atomic Emission Spectrometry-Fundamentals and Applications* (in Japanese), Kodansha Scientific, Tokyo (1986).
- (2) L. Sluyterman and O. Elgersma, *J. Chromatogr.*, 150 (1978) 17.
- (3) J. T. Wu, R.K. Pieper, L.H. Wu and J.I. Peters, *Clin. Chem.*, 35 (1989) 778.

CHAPTER 3

DEVELOPMENT HIGHLY SENSITIVE AND SELECTIVE DETECTION METHODS FOR THE REDOX COENZYME PYRROLOQUINOLINE QUINONE AND STUDY FOR ITS CHEMICAL SPECIATION IN BIOLOGICAL SAMPLES

3.1 Electrochemical Detection of Pyrroloquinoline Quinone Coupled with Its Catalytic Function by Liquid Chromatography

Increasing interest has been paid to the redox coenzyme pyrroloquinoline quinone (PQQ).¹⁻⁵ In the passed decade, a variety of methods of the detection and quantification of PQQ have been reported.^{1,2,4} In early studies, a number of enzymes such as bovine serum amine oxidase^{6,7}, methylamine dehydrogenase^{8,9} are reputed to contain covalently bound PQQ or PQQ derivatives. Recently, cofactors in several of those enzymes have been reexamined and proposed not to be PQQ.¹⁰⁻¹² These observations raise the question regarding the presence of PQQ in mammalian fluid. In contrast, however, biological importance^{2,13,14} and/or pharmaceutical activity of PQQ in mammalian^{15,16} are continued to be emphasized. These confused circumstances appear to be partly due to a lack of reliable, sensitive and selective analytical method of PQQ.

Gallop and colleagues reported a colorimetric assay of PQQ coupled with its redox-cycling reaction, where PQQ is reduced by glycine and the reduced PQQ is reoxidized by nitroblue tetrazolium to yield formazan to be detected.^{17,18,19} This method provides a high sensitive analysis of PQQ. However, this reaction is not specific to PQQ but has a broad spectrum for some other quinoid compounds, ascorbic and dehydroascorbic acids, and riboflavin.^{20,21} In addition, this cyclic reaction has revealed to compete with a linear reaction leading to unreactive oxazole condensation products.²² These problems seem to be overcome by employing some separation technique under suitable conditions. In this paper, we report a high-performance liquid chromatographic (HPLC) determination of PQQ with an

electrochemical detector coupled with a redox-cycling reaction by using ferricyanide ion ($\text{Fe}(\text{CN})_6^{3-}$) as an oxidant.

EXPERIMENTAL

Materials

Pyrrloquinoline quinone (PQQ) was obtained from Ube Industry and used as received. Swine serum (Cederlane, Canada) was commercially available. Table vinegar (from rice) and Milk were obtained from a grocery store. All other chemicals used were of analytical or LC grade.

Apparatus

Figure 1 shows two HPLC systems (Systems 1 and 2) employed here. System 1 consists of two HPLC pumps: Shimadzu LC-6AD (pump 1 for a mobile phase) and LC-9A (pump 2 for a reaction reagent solution), a Rheodyne 7125 injector with a 20 μl loop, a Yanagimoto VMD-101 voltammetric detector (with a glassy carbon electrode, an Ag/AgCl reference electrode, and a stainless tube counter electrode), and a Yokogawa 3056 chart recorder. In System 2, a Jasco FIU-200 peristaltic pump was used as pump 2 and a Shimadzu SPD-6A spectrophotometric detector was equipped. For separation, we employed two columns packed with octadecyl silylated silica gel: Cosmosil 5C-18AR (250 mm \times 4.6 mm i.d., Nacalai) and Capcell Pak C18 SG120A (250 mm \times 4.6 mm i.d., Shiseido) in Systems 1 and 2, respectively. A reactor was a Teflon coil tube with an inner diameter (i.d.) of 0.5 mm and immersed in a thermostat.

Sample preparation

Biological samples used here were table vinegar, milk, and swine serum. The samples except table vinegar were deproteinized by addition of 30 wt% of trichloroacetic acid (TCA). After centrifugation, excess TCA was removed from the supernatant by extraction with diethylether. After adjusting a pH value of the aqueous phase to be 0.5 with HCl to suppress the acid dissociation, PQQ was

extracted with isobutyl alcohol. In same case, the extracted solution was concentrated by evaporation. Control experiments for aqueous PQQ solution showed recovery of free PQQ over 80 %.

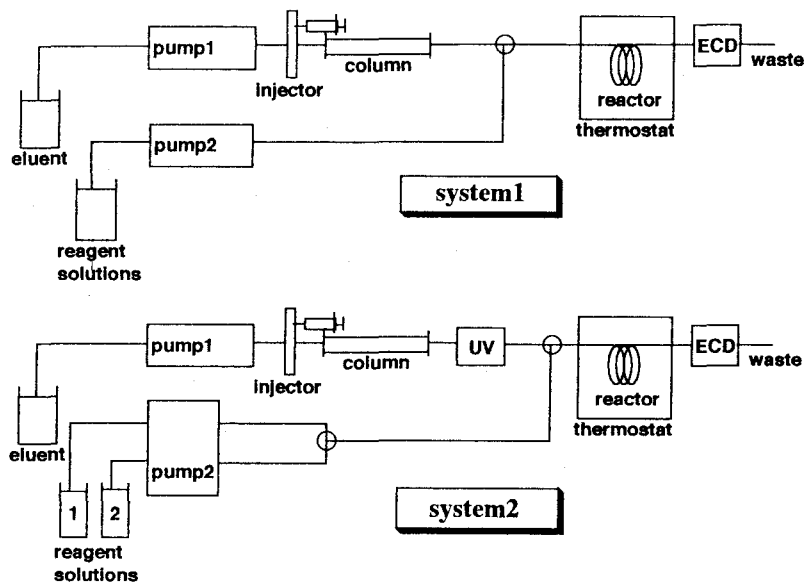


Figure 1 Schematic diagram of two on-line detection systems employed here See text for details. ECD: electrochemical detector, UV: ultraviolet spectrophotometric detector.

RESULTS AND DISCUSSION

Detection mechanism

Figure 2 illustrates the reaction utilized here. Certain amino acids are oxidized by PQQ to yield the reduced PQQ (quinol and/or aminophenol forms,

PQQ_{red}).^{1,5,17,23} Glycine is the most efficient reductant in this reaction among usual amino acids.¹⁷ The generated PQQ_{red} is reoxidized to PQQ by Fe(CN)₆³⁻. PQQ serves as a redox catalyst in the oxidation of glycine with Fe(CN)₆³⁻. Fe(CN)₆⁴⁻ is accumulated during the redox-cycling of PQQ. Thus, the electrochemical oxidation of the accumulated Fe(CN)₆⁴⁻ allows amplified detection of the catalyst, PQQ. Detail analysis of this reaction mechanism will be reported elsewhere.²⁴

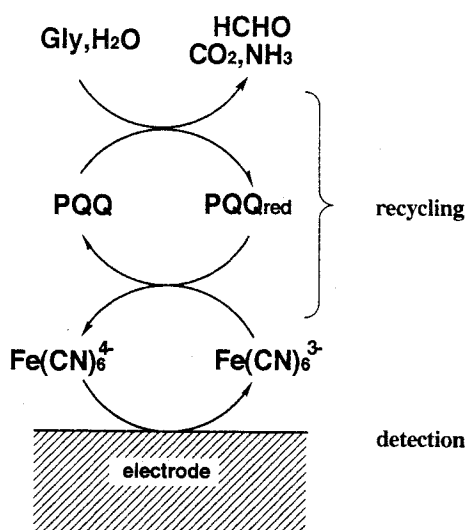


Figure 2 Redox-cycling reaction of PQQ employed in the amplified electrochemical detection of it. (See text for details.)

Optimum conditions of pyrroloquinoline quinone (PQQ) detection

Optimum conditions on System 1 were established as follows. A mixture of MeOH/0.06 M phosphoric acid (3/7 v/v)²⁵ containing K₃Fe(CN)₆ was used as a mobile phase with a flow rate of 1.0 ml min⁻¹. A glycine-ammonia buffer was used as a reaction reagent solution and mixed with the mobile phase in a post-column mode at a flow rate of 0.2 ml min⁻¹. The ammonia concentration was

adjusted so as to let a pH value of the final eluent to be 9.0. A working electrode potential was +0.5 V unless otherwise stated. We have found that glycine is directly reduced by $\text{K}_3\text{Fe}(\text{CN})_6$ even in the absence of PQQ with a very slow reaction rate. This is the reason why $\text{K}_3\text{Fe}(\text{CN})_6$ is not in the glycine-ammonia buffer but in the mobile phase.

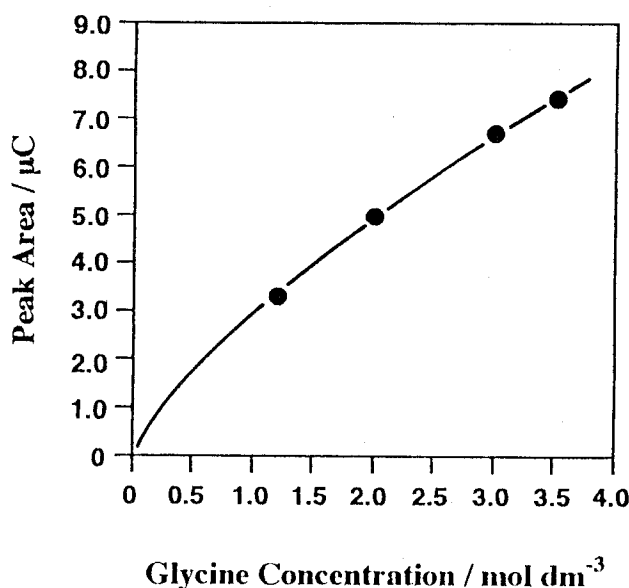


Figure 3 Relationship between the glycine concentration and the chromatographic peak area of PQQ detected with System 1 of Fig. 1.

The concentration of PQQ injected was 5 μM . $\text{K}_3\text{Fe}(\text{CN})_6$ concentration in mobile phase was 6.0 mM. A temperature of the reactor was 25 °C.

The observed peak area increased with the glycine concentration in the reagent solution, as shown in Fig. 3. In the following, 3.0 M glycine - 2.5 M ammonia buffer was used. The glycine concentration is close to the saturated one (ca. 3.5 M) and corresponds to 0.5 M as a final concentration after mixing. The

$K_3Fe(CN)_6$ concentration affected the peak area in a characteristic feature, as shown in Fig. 4. Although the peak area increased with the $K_3Fe(CN)_6$ concentration in the low concentration region below 5×10^{-6} M, $K_3Fe(CN)_6$ at higher concentrations over 1×10^{-5} M inhibited the net reaction. The reason has not been clarified yet. The optimum concentration of $K_3Fe(CN)_6$ was obtained as 6×10^{-6} M, which corresponds to 5×10^{-6} M as its concentration after mixing.

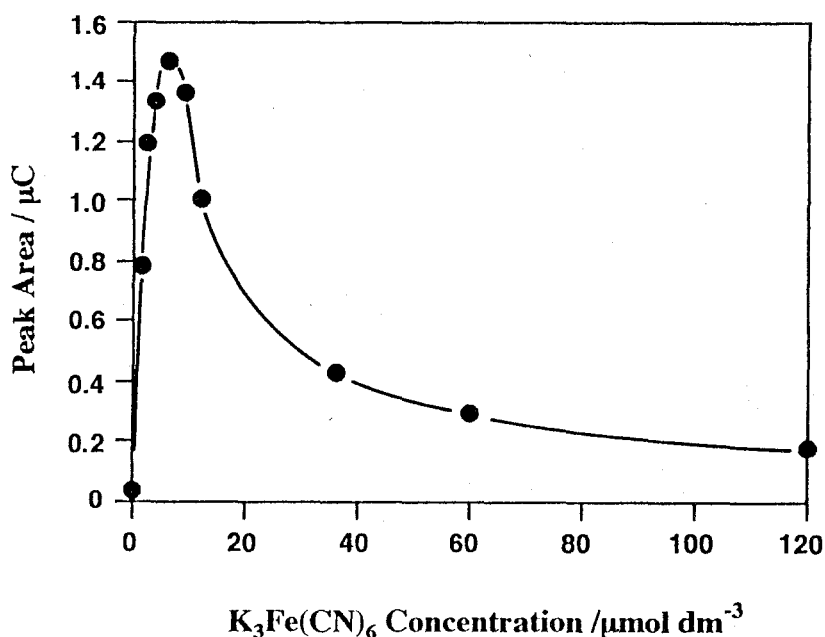


Figure 4 Effect of the $K_3Fe(CN)_6$ concentration on the chromatographic peak area of PQQ detected with system 1.

A glycine concentration in reagent solution was 3.0 M. A temperature of the reactor was 25 °C. The concentration of PQQ injected was 1 μM .

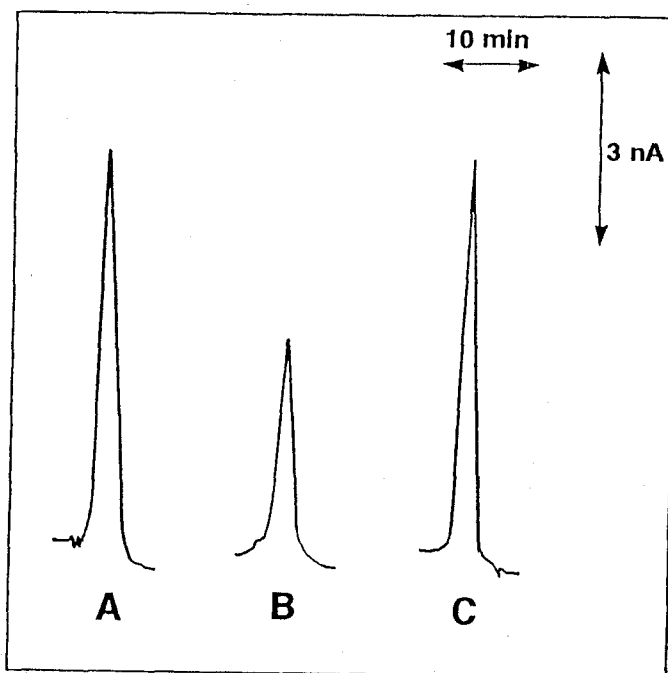


Figure 5 Chromatographic peaks of PQQ with three different electrochemical detection modes (A-C) under the identical separation conditions in System 1.

Peak A was obtained under the present optimized conditions: The concentrations of glycine in the reagent solution and $K_3Fe(CN)_6$ in the mobile phase were 3.0 M and 6.0 mM, respectively. 1 μ M of PQQ was injected. The working electrode potential was + 0.5 V and the reaction temperature was 25 °C. Peak B corresponds to a direct reductive detection of PQQ at the electrode potential of - 0.05 V. The mobile phase did not contain $K_3Fe(CN)_6$ and pure water was used instead of the reagent solution. 50 μ M of PQQ was injected. The others are as in A. Peak C was detected in the absence of $K_3Fe(CN)_6$ in the mobile phase. The other conditions are the same as those in A. Under these conditions, the dissolved oxygen would serve as an oxidant in the place of $Fe(CN)_6^{3-}$. 50 μ M of PQQ was injected.

We employed a coiled Teflon tube with 0.5 mm i.d. as a reactor. The observed peak area increased linearly with the tube length up to 17.5 m, which corresponds to ca. 3 min of the reaction time and was estimated to be the optimum one. Prolonged length beyond 17.5 m resulted in a saturation of the peak increase as well as serious broadening of the peak. The saturation phenomenon is attributable to the oxazole adduct formation between PQQ and glycine. The oxazole adduct is inactive in this redox-cycling reaction. An increase in the reaction temperature gave rise to an exponential increase in the peak area, though the noise increased concomitantly. The maximum S/N ratio was attained around 45 °C. An apparent activation energy under the present conditions was estimated to be 12 Kcal mol⁻¹.

Figure 5 demonstrates effectiveness of the redox-cycling reaction of PQQ in its detection. Peak A is part of a chromatogram of PQQ obtained under the present optimized conditions. PQQ itself is electrochemically active²⁶ and hence can be directly and *reductively* detected at -0.05 V, as shown in Peak B in Fig. 5. Note here that the injected amount of PQQ in Peak B (and Peak C) is 50 times that in Peak A. The amplification factor or the apparent "turnover number" expressed as an area ratio of Peak A/Peak B at a given amount of PQQ was 91 at a reaction temperature of 25 °C. By considering the temperature effect of the reaction, the amplification factor of 360 would be expected at 45 °C. Peak C in Fig. 5 shows an *oxidative* detection of PQQ_{red} which has been generated during the reaction with glycine in the absence of K₃Fe(CN)₆. The peak area is about two times larger than that of Peak B. This phenomenon would be attributed to a relatively slow oxidation of PQQ_{red} by dissolved oxygen to yield hydrogen peroxide, which can be detected at 0.5 V together with PQQ_{red}. Comparison of Peaks A and C proves that K₃Fe(CN)₆ is preferred over dissolved oxygen as an oxidant in the redox-cycling reaction of PQQ. A calibration curve for the PQQ determination yielded a good linearity with $r = 0.998$ over the range from 1×10^{-7} M to 6×10^{-6} M of PQQ for a 20 μ l-injection and at 25 °C of the reaction temperature. The detection limit was 0.2 pmol at $S/N = 3$. This sensitivity is 30 times higher than that with

spectrophotometric detection at 249 nm under identical separation conditions. Unfortunately, PQQ gives a relatively broad peak under the present separation conditions.²⁵ Some improvement in the separation conditions might be desired. The large amplification of the PQQ signal might cause the depression of the $\text{Fe}(\text{CN})_6^{3-}$ concentration at elevated amounts of PQQ. By considering more than a hundred times dilution during HPLC separation, the depression effect is negligible in this concentration range of PQQ. For higher concentrations of PQQ over 1×10^{-5} M, however, a corresponding higher concentration of $\text{K}_3\text{Fe}(\text{CN})_6$ can be used.

Application to biological samples

The presence of PQQ in mammalian samples and foods has been recently argued.^{14,17,18,20,27,28} In order to get some information concerning this point, we have applied our technique to the detection of free PQQ in table vinegar, milk, and swine serum. System 2 was constructed here in order to detect PQQ simultaneously with electrochemical and spectrophotometric detectors. This dual detection system will work for more accurate identification of chromatographic peaks. A mixture of MeOH/0.056 M phosphoric acid (1/3 v/v) was pumped at a flow rate of 1.0 ml min^{-1} in this experiment. Reaction reagent solutions 1 and 2 containing 3.0 M glycine - 2.5 M NH_3 buffer and 3.65×10^{-5} M $\text{K}_3\text{Fe}(\text{CN})_6$, respectively, were mixed with the mobile phase at 0.2 ml min^{-1} . The detection limit on System 2 was 2 pmol with the electrochemical detection for a 20 μl -injection. The wavelength of the UV detection was 249 nm.

It has been reported that PQQ can be detected in vinegar with biological assay.²⁰ Although we tried to detect free PQQ in vinegar, no evidence was provided for the presence of free PQQ in a 100-fold concentrated vinegar. This might be attributable to in part lactone and/or dimer formation during the acid treatment and succeeding concentration.²⁹ However, after addition of PQQ (2.0×10^{-6} M) to a vinegar sample, the PQQ was successfully detected. The resultant chromatograms with electrochemical and spectrophotometric detectors are given in

Fig. 6. When the reaction reagent solution 1 was replaced with glycine-free ammonia buffer, the electrochemically detected peak at an elution time of 15.0 min disappeared completely, while the spectrophotometrically detected chromatogram remained unchanged. These observations assisted in peak identification for PQQ. Comparison of the two chromatograms provides a clear evidence of the preference of the present electrochemical detection over the spectrophotometric one.

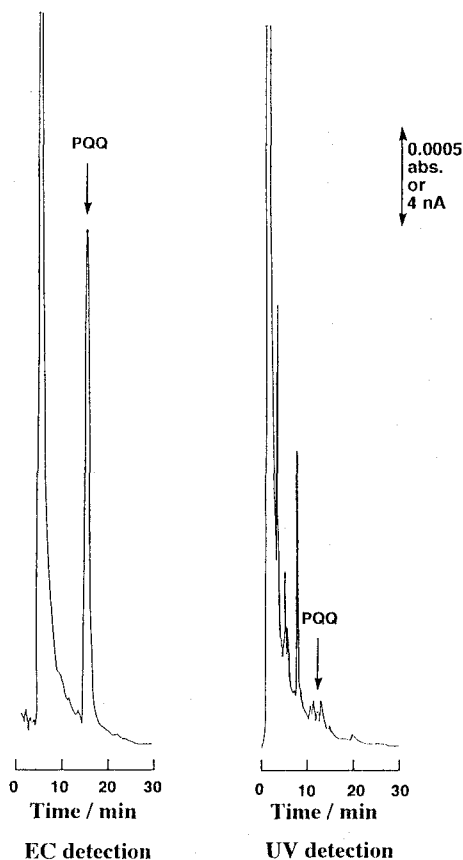


Figure 6 Chromatograms of a PQQ spiked table vinegar sample obtained with on-line detections of the electrochemical method and ultra violet spectrophotometry at 249 nm in System 2 in Fig. 1.

See text for details concerning the pretreatment of the sample and the electrochemical detection coupled with the redox-cycling reaction. The arrows indicate the elution time at which free PQQ should be observed.

Stability of PQQ in the vinegar sample was also studied. The free PQQ concentration was found to decrease with the elapsed time (incubation time) from the addition to the vinegar sample, as shown in Fig. 7. Most probably, the free PQQ is gradually converted into its derivatives.

For a 100-fold concentrated milk sample or a 15-fold concentrated swine serum sample, no peak was observed at the elution time of free PQQ. Once PQQ was spiked into a milk sample, a peak of PQQ was clearly detected and the peak height decreased with the incubation time as in the case of table vinegar. However, the peak height was much smaller than that expected from the added amount. This might be attributable to higher reactivity or adsorptivity of PQQ toward milk components.

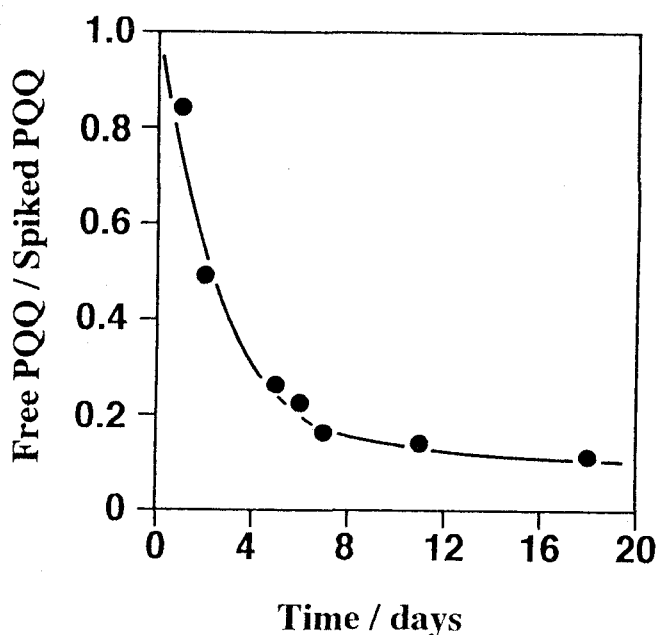


Figure 7 Time-dependent change in the free PQQ concentration in a vinegar sample spiked with 50 mM of PQQ.

The PQQ concentration was determined with the electrochemical detection in system 2. Separation and detection conditions were as in Fig. 5.

SUMMARY

Coenzyme pyrroloquinoline quinone (PQQ) was electrochemically detected with high sensitivity and high selectivity by employing its redox catalytic function in reversed-phase high performance liquid chromatography. This catalytic reaction involves oxidative decarboxylation of glycine by PQQ and the reoxidation of the reduced PQQ by $\text{Fe}(\text{CN})_6^{3-}$ to accumulate $\text{Fe}(\text{CN})_6^{4-}$, of which the electrochemical detection allows amplified detection of PQQ. Increase in two order of magnitude of the current was achieved as compared with a direct reductive detection, at a reaction time of 3 min and a reaction temperature of 25 °C. The detection limit was 0.2 pmol (10^{-8} M, 20 μl). The present method was applied to quantification of PQQ in table vinegar, milk, and swine serum.

REFERENCES

- (1) J.A. Duine, J. Frank, and J.A. Jongejan, in "*Advances in Enzymology* " , ed. A. Meister, vol. 59, p. 169, John Wiley & Sons, New York , 1987.
- (2) J.A. Jongejan and J.A. Duine eds., "*PQQ and Quinoproteins*", Kluwer Academic Publishers, Dordrecht , 1989.
- (3) J.A. Duine and J.A. Jongejan, *Annu. Rev. Biochem.*, 58 (1989) 403.
- (4) R.A. van der Meer, B.W. Groen, M.A.G. van Kleef, J. Frank, J.A. Jongejan, and J.A. Duine, in "*Methods in Enzymology* " ed. M.E. Lidstrom, vol. 188, p. 260, Academic Press, San Diego, 1990.
- (5) Y. Ohshiro and S. Itoh, *Bioorg. Chem.*, 19 (1991) 169.
- (6) C.L. Lobenstein-Verbeek, J.A. Jongejan, J. Frank, and J.A. Duine, *FEBS Lett.*, 170 (1984) 305.
- (7) M. Ameyama, M. Hayashi, K. Matsushita, E. Shinagawa, and O. Adachi, *Agric. Biol. Chem.*, 48 (1984) 561.
- (8) W.S. McIntire and J.T. Stults, *Biochem. Biophys. Res. Commun.*, 141 (1986) 562.
- (9) F.M.D. Vellieux and W.G. Hol, *FEBS Lett.*, 255 (1989) 460.
- (10) S.M. Janes, D. Mu, D. Wemmer, A.J. Smith, S.Kaur, D. Maltby, A.J. Burlingame, and J.P. Klinman *Science*, 248 (1990) 981.
- (11) N. Ito, S.E.V. Phillips, C. Stevens, Z.B. Ogel, M.J. McPherson, J.N. Keen, K.D.S. Yadav, and O.F. Knowles, *Nature*, 350 (1991) 87.
- (12) W.S. McIntire, D.E. Wemmer, A. Chistoserdov, and M.E. Lidstrom, *Science*, 252 (1991) 817.
- (13) P.M. Gallop, M.A. Paz, R. Flückiger, and E. Henson, Abstract: 2nd International Symposium on PQQ and Quinoproteins, 1991, Nov. (Yamaguchi, Japan), p. 40, 1991.
- (14) J. Killgore, C. Smidt, L. Duich, N. Romero-Chapman, D. Tinker, K. Reiser, M. Merko, D. Hyde, and R.B. Rucker, *Science*, 245 (1989) 850.
- (15) H. Nishigori, O. Ishida, and I.O. Umeda, Abstract: 2nd International Symposium on PQQ and Quinoproteins, 1991, Nov. (Yamaguchi, Japan), p. 39, 1991.

- (16) H. Nishigori, M. Yasunaga, M. Mizumura, J.W. Lee, and M. Iwatsuru, *Life Sci.*, 45 (1990) 593.
- (17) R. Flückiger, T. Woodtli, and P.M. Gallop, *Biochem. Biophys. Res. Commun.*, 153 (1988) 353.
- (18) M.A. Paz, P.M. Gallop, B.M. Torrelio, and R. Flückiger *Biochem. Biophys. Res. Commun.*, 154 (1988) 1330.
- (19) M.A. Paz, R. Flückiger, A. Boak, H.M. Kagan, and P.M. Gallop, *J. Biol. Chem.*, 266 (1991) 689.
- (20) R.A. van der Meer, B.W. Groen, J.A. Jongejan, and J.A. Duine, *FEBS Lett.*, 261 (1990) 131.
- (21) A. Schvenberg and R. Moubacher, *Chem. Rev.*, 50 (1952) 261.
- (22) M.A.G. van Kleef, J.A. Jongejan, and J.A. Duine, *Eur. J. Biochem.* 183 (1989) 41.
- (23) S. Itoh, N. Kato, Y. Ohshiro and T. Agawa, *Tetrahedron Lett.*, 25 (1984) 4753.
- (24) T. Mori, K. Kano, Y. Esaka, B. Uno and M. Goto, Presented at the 37 th Annual Meeting on Polarography and Electroanalytical Chemistry, 1991, Nov. (Kyoto, Japan) : Abstract : *Rev. Polarogr*, 37 (1991) 29.
- (25) K. Kano, B. Uno, C. Kawasaki, K. Horiki, and S. Kawai, *Anal. Sci.*, 7 (1991) 737.
- (26) K. Kano, B. Uno, T. Kubota, and M. Senda, *Bioelectrochem. Bioenerg.*, 23 (1990) 227; idem. *ibid.*, 24 (1990) 193.
- (27) M. Ameyama, E. Shinagawa, K. Matsushita, and O. Adachi *Agric. Biol. Chem.*, 49 (1985) 699.
- (28) M.A. Paz, R. Flückiger, E. Henson, and P.M. Gallop, p.131 of ref.2.
- (29) P.M. Gallop, E. Henson, M.A. Paz, S.L. Greenspan, and R. Flückiger, *Biochem. Biophys. Res. Commun.*, 163 (1989) 755.

3.2 Separation of Amino Acid-oxazole Derivatives of the Redox Coenzyme Pyrroloquinoline Quinone by Capillary Zone Electrophoresis

Pyrroloquinoline quinone (PQQ, see Fig. 1) was discovered in bacteria as a novel redox coenzyme of non-flavin or nicotinamide-dependant dehydrogenases in 1979 ^{1,2}. In early studies, several enzymes such as bovine serum amine oxidase ^{3,4}, methylamine dehydrogenase ^{5,6} were reputed to contain covalently bound PQQ and PQQ derivatives. Further, the occurrence of free PQQ in mammalian fluids and also, for example, in serum and milk was proposed ^{7,8}. Recently, however, topa quinone ^{9,10} and tryptophan tryptophyl quinone ¹¹ have been proved to be the organic cofactors of copper-containing amine oxidases and methylamine dehydrogenase, respectively. In turn, the occurrence of PQQ in mammalian fluids has been questioned, while the biological importance ¹²⁻¹⁴ and pharmaceutical activity of PQQ in mammalian ^{11,15} continue to be emphasized.

Such confused circumstances appear to be partly due to a lack of reliable, sensitive and selective analytical method of PQQ. We have reported a high performance liquid chromatographic (HPLC) determination of free PQQ with an electrochemical detector coupled with the amplification by a redox-cycling reaction of PQQ with glycine ⁷ and applied it to the determination of free PQQ in milk and swine serum ¹⁶. However, no free PQQ was detected. In contrast, the immediate disappearance of free PQQ added to those biological fluids has been proved. This suggests that most of PQQ in such biological media, if any, would be converted to some redox-inactive derivatives. Hence there seems to be an urgent need to develop an analytical method to speciate PQQ and its derivatives in biological fluids in order to provide some breakthrough in this confused field.

PQQ is known to be very reactive toward nucleophilic reagents ¹⁷⁻¹⁹. Amino acids can be considered to be the most important and widely distributed compounds among many nucleophilic compound in biological fluids in view of the nucleophilic reaction toward PQQ. As shown in Fig. 1, free PQQ reacts readily with amino acids under aerobic conditions to yield corresponding oxazole

derivatives (oxazolopyrroloquinolines, OPQs), as the final products 19-22. The OPQs show variety in the structure of the R groups. OPQs with a hydrogen atom and amino acid residues as the R group will be called OPQ1 and OPQ2, respectively, in this paper. All these OPQs derivatives have no redox-catalytic activity. Our hypothesis here is that the OPQ(s) is the major product(s) from PQQ in reactions in biological fluids.

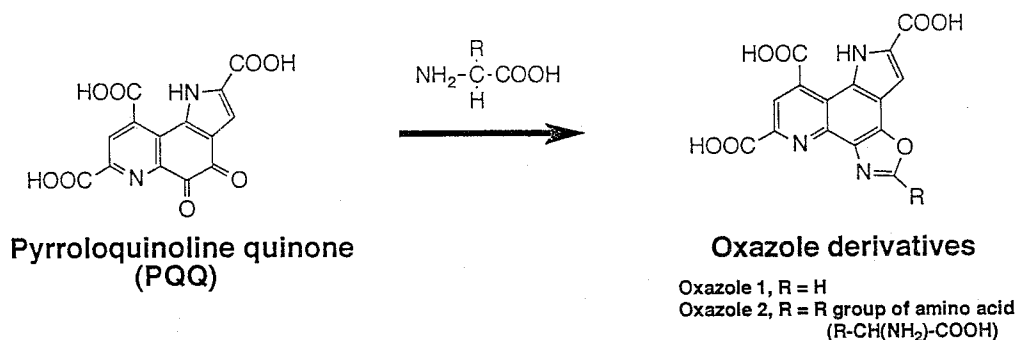


Figure 1 Formation of amino acid-oxazole derivatives (oxazolopyrroloquinolines, OPQs) of PQQ.

Although reversed-phase (RP) HPLC with gradient elution ²¹ and ion exchange chromatography ²² were reported for the analysis of the OPQs, they might not necessarily be suitable for the separation of OPQs. The reasons are that the OPQs are strongly charged molecules owing to the three carboxyl groups and the acidic and basic amino acid residues and that their structures are only slightly different from each other (Fig. 1). RP-HPLC would not be suitable for the separation of such charged molecules and structurally related compounds with an identical charge might not be separated completely by ion-exchange chromatography.

Capillary zone electrophoresis (CZE) has a very high resolution for charged samples. Our aims in this work were to separate all OPQs by employing CZE with good resolution and to apply the method to the product analysis of the reaction

between PQQ and each amino acid. Further, the speciation of PQQ in biological fluid will be discussed based on analyses of PQQ-spiked bovine serum sample.

EXPERIMENTAL

Materials

Pyrroloquinoline quinone (PQQ) was obtained from Ube Industry (Tokyo, Japan) and used as received. Bovine serum was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used were analytical-reagent grade.

Apparatus

Electrophoretic separation was performed in a fused-silica tube (GL Science, Tokyo, Japan) of 0.05-mm I.D. and a length of 550 mm, with an effective length for separation, i.e., the length from the injection end to the detection cell, of 300 mm unless otherwise noted. A Matsusada Precision Devices (Kusatsu, Japan) Model HCZE-30PNO high-voltage d.c. power supply, which can deliver high voltage up to 30 kV, was employed to supply the potential across the capillary. For UV-Vis spectrophotometric detection, a Jasco 875-UV detector (Tokyo, Japan) was employed. The detection wavelength was set at 250 or 420 nm. The flow cell was modified for capillary electrophoresis in our laboratory. A Shimadzu (Kyoto, Japan) Chromatopac C-R6A was used for data processing.

Sample preparations

OPQs were generated by incubation of PQQ (0.01 M) and each amino acid (0.004 g/ml) in 200 mM acetate buffer (pH 3.5) or 100 mM phosphate buffer (pH 7.9) at a volume ratio of 1:9. The incubation period was about 2 days at a room temperature. OPQ1 from glycine and OPQ2 from valine were purified by thin-layer chromatography with silica gel plates (Kieselgel 60 F₂₅₄, Merck) in a mixed solvent of methanol-chloroform-water(70:30:7). These were used as the standard samples. In routine analysis, the reaction mixtures were subjected to CZE analysis without any purification.

A bovine serum sample was pretreated as follows. The sample was applied to an open column packed with DEAE-celullose and washed with 200 ml of 0.1 M phosphate-Tris buffer (pH 6.0). The resin at the top of the column was removed and suspended in 30 ml of 0.5 M NaCl solution. After adjusting the pH of the solution to 0.5 with HCl to suppress the acid dissociation, adsorbed compounds were removed from the resin by shaking for 15 min and then extracted with isobutyl alcohol. The extracted solution was concentrated by evaporation. Control experiments for the standard oxazole 1 added to bovine serum showed a recovery of more than 95 %.

Procedure

Just before each run, a separation capillary tube was purged with 0.1 M NaOH for 2 min and then with a desired buffer for 3 min by using an aspirator. The two ends of the tube were then dipped into two separated 1.5-ml reservoirs filled with the same buffer. The end at which samples were introduced was connected with a platinum electrode to positive high voltage, while the other end was connected with a platinum electrode to ground. Samples were introduced by siphoning at a height of 15 cm for a 5 to 10-s period.

RESULTS AND DISCUSSION

CZE separation of oxazolopyrroloquinolines (OPQs)

In preliminary experiments in which electrophoresis of the standard samples (OPQ1 from glycine and OPQ2 from valine) was carried out with phosphate buffer containing no modifier, the OPQs did not exhibit the peak or, even if a peak was detected, the electropherograms resulted in very poor reproducibility. We ascribe this phenomenon to the adsorption of the analytes on the capillary wall. To overcome such adsorption effect, we examined several organic solvents as modifiers²³. In this work, 10 mM phosphate buffer (pH 8.0) containing 5 or 10 % (v/v) of a certain organic solvent was employed as an electrolyte solution, in which the three carboxyl groups in the OPQs are expected to be acid dissociated, as in the case of PQQ^{24,25}. The addition of any one of dimethyl sulfoxide (DMSO), methanol (MeOH) and acetonitrile (ACN) was found to be efficient to obtain the reproducible peaks of the oxazoles. From the point of view of the complete

separation of the OPQs (see below), however, DMSO was most preferred as a modifier for high resolution. The theoretical plate number (N) was calculated to be 2.75×10^5 for OPQ1 based on the equation: $N = l^2/2Dt$, where l , D and t are the separation distance, the diffusion coefficient and the migration time of a sample, respectively²⁶. In the above calculation, the D value of PQQ ($3.9 \cdot 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ²⁴) was used. When MeOH was used as a modifier, a somewhat lower resolution was obtained with DMSO, and ACN gave an even lower resolution. This might be explained in terms of a relatively small value of D in the DMSO mixed solvent owing to its high viscosity and/or its large number of solvating molecules compared with the others. Electroosmotic flow-rate was virtually independent of the organic solvents used at such low concentrations, although at a concentration above 20 % a marked effect on the flow-rate was observed, as has been reported elsewhere²⁷.

Reactions of PQQ with amino acids

Product analyses of the reactions of PQQ with several amino acids were performed by the present method together with absorption spectroscopy. Peak identification was performed by addition of standard samples and comparison of two electropherograms detected at 250 and 420 nm. OPQs are reported to exhibit a characteristic adsorption band around 420 nm²¹, at which the absorption of PQQ and other adducts is very weak compared with that at 250 nm.

Figure 2 shows electropherograms of the reaction mixtures (pH 7.9) of four amino acids detected at 250 nm and Table 1 summarizes the yields of OPQ1 and OPQ2 generated from several amino acids. Although the reaction products depend greatly on the amino acids and the reaction pH, we divided the amino acids into five classes (see Table 1 and below).

A typical amino acid in class 1 is glycine, with which the reaction led to the formation of OPQ1 exclusively (Fig. 2a). The absorption spectrum of the reaction mixture gave a characteristic absorption maximum of OPQ1 around 420 nm (Fig. 3, curve 1). Virtually identical results were obtained at pH 3.5 and 7.9. A similar behavior was observed for the reaction with tryptophan (Table 1). These results are in close agreement with those of other workers²⁰⁻²². Threonine can be included in this class, though OPQ2 was detected as a minor product.

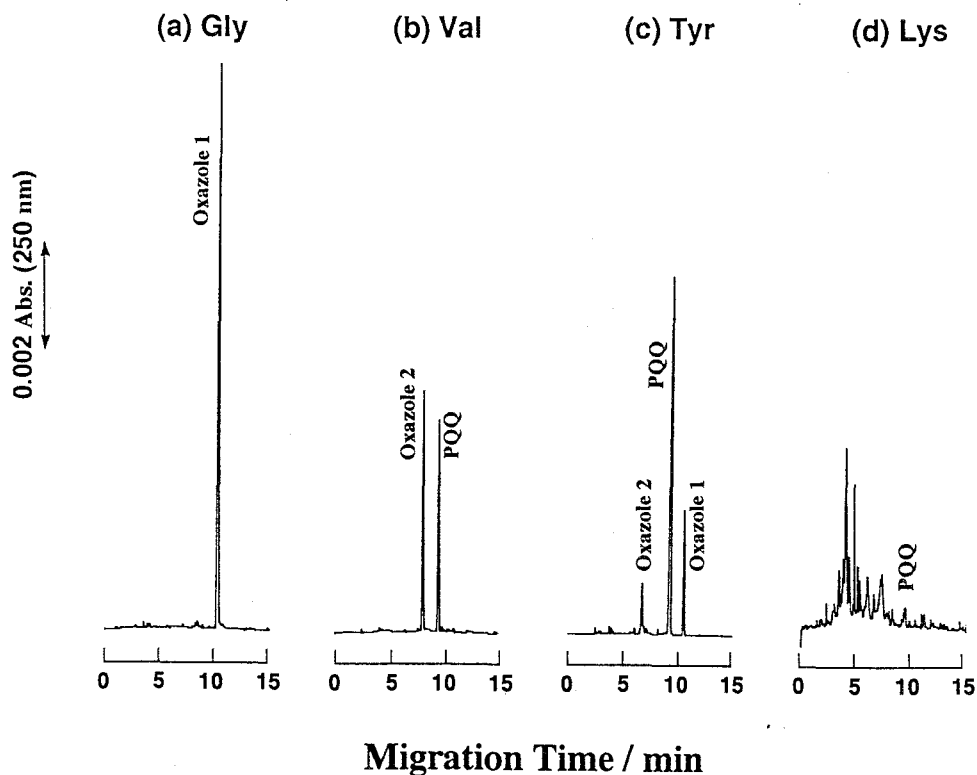


Figure 2 Electropherograms of reaction solutions of PQQ with (a) glycine, (b) valine, (c) tyrosine, and (d) lysine at pH 7.9 for 48 hr at room temperature.

Electrolyte solution: 10 mM phosphate buffer (pH 8.0) containing 5% (v/v) dimethyl sulfoxide, capillary: 550 mm \times 0.05 mm i.d. (300 mm as an effective length), separation voltage: 23 kV, current: 17 μ A, injection (siphoning) : 15 cm, 7 s, detection wavelength: 250 nm.

In the reaction with valine, OPQ2 was predominantly generated, while some PQQ remained unchanged (Fig. 2b). The absorption spectrum of the reaction mixture exhibited an absorption band around 420 nm (Fig. 3, curve 2). The product was virtually independent of the reaction pH. This class (class II) involves alanine, leucine, isoleucine, phenylalanine, asparagine, glutamine, aspartic acid, and glutamic acid (Table 1). For some of these amino acids, OPQ1 was also formed as a minor product. Methionine and histidine yielded the corresponding OPQ2s as major products at pH 3.5 and 7.9 and can therefore be assigned to class II. However, there were several minor products different from the OPQs.

With tyrosine, both OPQ1 and OPQ2 were detected (Fig. 2c). but the ratio of OPQ1 to OPQ2 depended greatly on the reaction pH: at pH 3.5 the OPQ2 was generated preferentially, whereas at pH 7.9 OPQ1 was the major product. A very similar behavior was observed for serine. Such a pH-dependent reaction of serine has been reported firstly by Adachi²⁸ and then in detail by Itoh *et al*²².

Amino acids that did not yield any detectable OPQs were assigned to class IV. Lysine exhibited a very complicated electropherogram (Fig.2d), indicating the existence of several reaction pathways. The absorption spectrum of the reaction mixture was completely different from that of glycine. Although the formation of a small amount of OPQs is suggested by a shoulder around 420 nm (Fig. 3, curve 3), there appeared no remarkable peak in an electropherogram detected at 420 nm (not shown). The reaction with arginine resulted in the formation of one major adduct. The adduct, however, seems to be different from OPQs, because the detection at 420 nm showed only a small peak and the absorption spectrum of the mixture was completely different from that of glycine (Fig. 3, curve 4).

Class V involves proline, hydroxyproline, cysteine, cystine. These amino acids did not give adducts, though cysteine is oxidized to cystine by PQQ²⁹

Table 1 Yields of OPQ1 and OPQ2 in the reactions of PQQ with several amino acids at pH 3.5 and 7.9

Incubation: 48 hr at room temperature. Lys and Arg (class IV) yielded no detectable oxazoles but some adducts different from OPQs. Pro, Hyp, Cys, and Cys-Cys (class V) yielded no adducts.

Amino Acids	Yield% (pH 3.5)		Yield% (pH 7.9)		Class
	oxazole 1	oxazole 2	oxazole 1	oxazole 2	
Gly	100	-	100	-	I
Trp	97	-	92	-	I
Thr	75	22	84	12	I
Val	-	81	<1	67	II
Ala	3	87	13	72	II
Leu	-	93	6	72	II
Ile	-	84	3	61	II
Phe	3	97	2	83	II
Asn	-	95	6	70	II
Gln	-	97	3	90	II
Asp	-	85	-	38	II
Glu	4	92	6	50	II
Met	-	68	-	32	II
His	-	94	-	27	II
Tyr	-	73	23	12	III
Ser	2	94	91	8	III

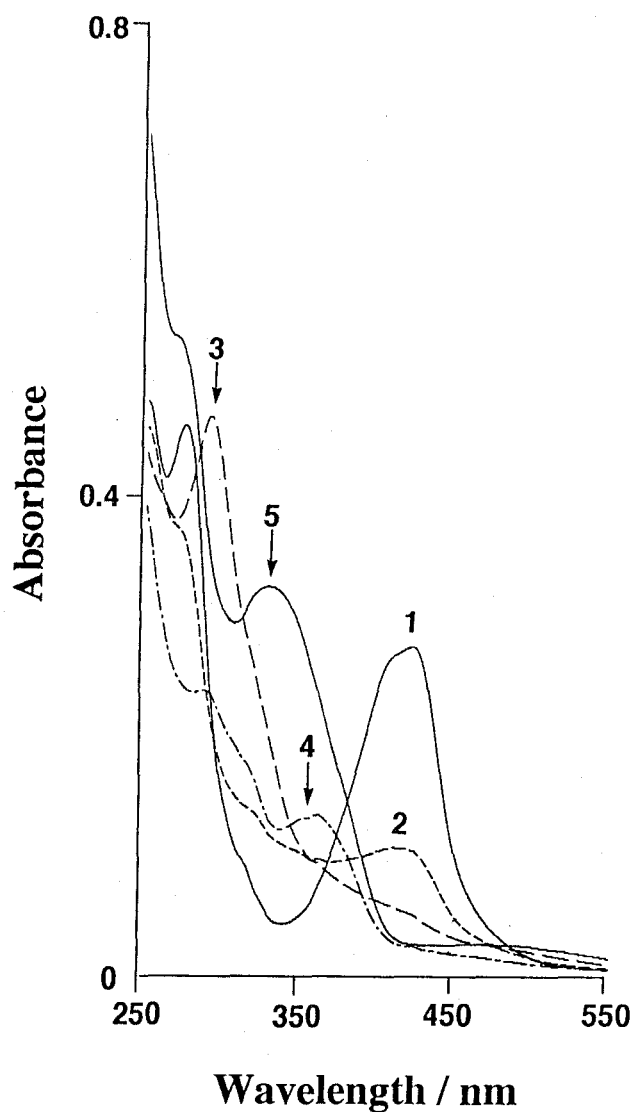


Figure 3 Absorption spectra of reaction solutions of PQQ with (1) glycine, (2) valine, (3) lysine, and (4) arginine and (5) of free PQQ.

Reaction conditions: PQQ (0.01 M) : amino acid (0.004 g/ml in 100 mM phosphate buffer, pH 7.9) = 1 : 9, incubated for 48 hr at room temperature. Before measurement, reaction solutions were diluted with 5-fold volume of 10 mM phosphate buffer (pH 7.9).

The results suggest that there are two major competing reaction pathways to give OPQ1 and OPQ2s. We propose a mechanism shown in Fig. 4 for the formation of OPQs. The reaction between PQQ and amino acids has been reported to proceed via an ionic mechanism involving carbinolamine-type intermediates which are dehydrated to give iminoquinones^{9,28}. When C $_{\alpha}$ -C $_{\beta}$ bond cleavage followed by decarboxylation proceeds from the iminoquinones, OPQ1 is generated as a major product. The pathway is similar to that proposed by Van Kleef *et al.*²¹. In contrast, when decarboxylation is facilitated in the iminoquinone prior to the C $_{\alpha}$ -C $_{\beta}$ bond fission, OPQ2 will be predominantly produced. This reaction pathway has already been proposed by Ohshiro and co-workers^{19,22}.

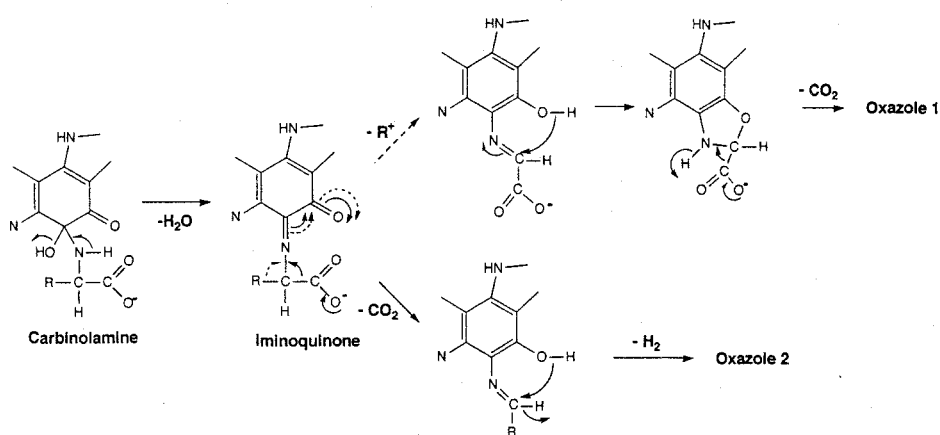


Figure 4 Proposed mechanism of the formation of OPQ1 and OPQ2s.

A predominant path seems to depend on the amino acid residues and also the reaction conditions. For the amino acids in class I (except glycine), the C_{α} - C_{β} bond cleavage would be facilitated, but the decarboxylation is the path for the amino acids in class II. With serine and tyrosine (class III), base-catalyzed C_{α} - C_{β} bond fission would occur rather than the decarboxylation at an elevated pH ²². Lysine and arginine in class IV and histidine in class II are basic in nature and converted into some adducts different from OPQs in major or minor processes. These amino acids have two nucleophilic groups in each molecule. In view of this, it is interesting to refer to the reaction of ethylenediamine ³⁰ and aminoguanidine ³¹ with PQQ, in which pyrazine and triazine derivatives are, respectively, generated.

Figure 5 illustrates an electropherogram of a mixture of the reaction solutions of PQQ with most of the amino acids in classes I, II, and III (glycine, tryptophan, threonine, valine, alanine, leucine, phenylalanine, asparagine, glutamine, aspartic acid, glutamic acid, histidine, tyrosine, and serine) except isoleucine and methionine. Each peak was identified by the addition of the corresponding OPQ. The OPQ2 from isoleucine gave a peak at a position identical with that of the OPQ2 from leucine owing to their strong structural similarity. The peak of the OPQ2 from methionine was overlapped with that of glutamine. The amino acids in class IV would make electropherograms complicated because they yield many kinds of minor products, as mentioned above.

Baseline resolution of most of the oxazole derivatives was obtained, except two OPQ2 from threonine and valine within ca. 25 min. The present separation appears to be much superior to those obtained with HPLC in terms of the resolution and the separation time. The detection limit for OPQ1 was ca. 50 fmol (S/N = 5, 10 μ M, ca. 5-nl injection).

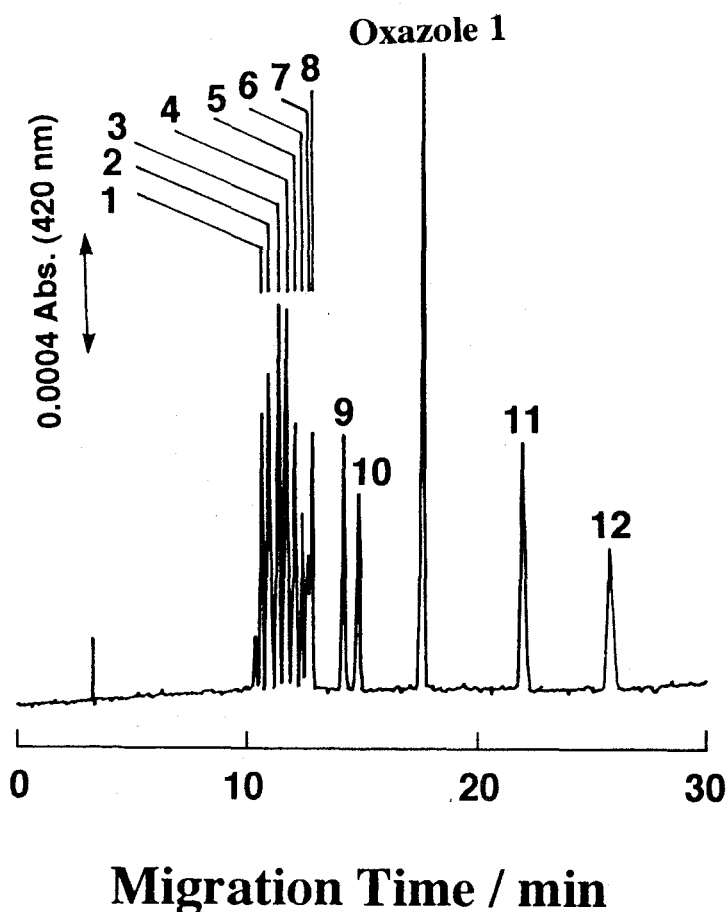


Figure 5 Electropherogram of a mixture of reaction solutions of PQQ with several amino acids at pH 3.5.

Electrolyte solution: 10 mM phosphate buffer (pH 8.0) containing 5 % (v/v) dimethyl sulfoxide, capillary: 700 mm \times 0.05 mm i.d. (450 mm as an effective length), separation voltage: 25 kV, current: 14 μ A, injection (siphoning) : 15 cm, 7 s, detection wavelength: 420 nm. Peak 1-12: OPQ2s generated from tyrosine, histidine, phenylalanine, leucine, glutamine, asparagine, threonine, valine, serine, alanine, glutamic acid, and aspartic acid, in turn.

Analysis of PQQ-spiked bovine serum and native bovine serum

PQQ and OPQs in PQQ-spiked bovine serum were determined using the present CZE method. The pretreatment was carried out at about 48 hours after the PQQ addition. An electropherogram of PQQ-spiked bovine serum sample is given in Fig. 6. For this sample, the PQQ concentration added to serum was $5 \cdot 10^{-6}$ M and a 30-fold concentration was carried out in the preparation. OPQ1 was observed as a major peak component. The peak of OPQ was identified by adding the standard OPQ1 to the pretreated serum sample. The spectrum of the PQQ-spiked sample also supported the oxazole formation (not shown). When PQQ was spiked at a higher concentration of $2 \cdot 10^{-4}$ M, some OPQ2s together with the unreacted free PQQ were detected as minor peaks.

These results indicated that most of the PQQ existing in serum as the oxidized form is converted into OPQ1 during the course of the reaction with free amino acids. The reason why OPQ1 is a major product seems to be that the rate of the formation of oxazole 1 from glycine is much faster than that of OPQ2s from other amino acids.

For a native (non PQQ-spiked) bovine serum sample, however, no OPQs were found, even though a *ca.* 100-fold concentration was performed in the pretreatment. Hence the concentration of PQQ-OPQs in the bovine serum might be expected as less than 10^{-7} M, if any. For further studies, some method of more effective and selective preconcentration seems to be required.

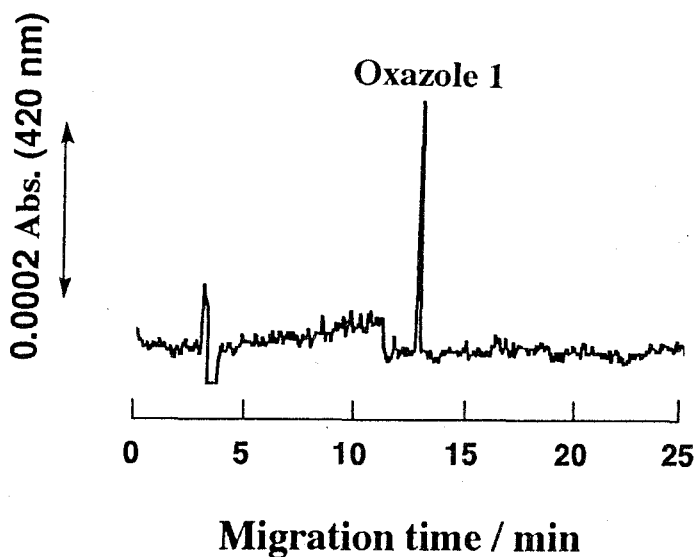


Figure 6 Electropherogram of PQQ-spiked bovine serum sample.

Electrolyte solution: 10 mM phosphate buffer (pH 8.0) containing 5% (v/v) dimethyl sulfoxide, capillary: 550 mm \times 0.05 mm i.d. (300 mm as an effective length), separation voltage: 17 kV, current: 13 μ A, injection (siphoning) : 15 cm, 7 s, detection wavelength: 250 nm. See text for pretreatment of the sample.

SUMMARY

Condensation products (oxazole derivatives) from the reaction of coenzyme pyrroloquinoline quinone (PQQ) with several α -amino acids have been successfully separated by capillary zone electrophoresis. Addition of a certain organic solvent such as dimethyl sulfoxide into the electrolyte solution is essential for the reproducible and complete separation. The organic modifier appears to prevent the oxazole derivatives from adsorbing on the capillary wall. Product analysis of the condensation reactions of PQQ with amino acids was performed by this method and the mechanism is discussed briefly. PQQ-spiked bovine serum was also analyzed. Unsubstituted type 1 oxazole derivative was detected predominantly. This result suggests that most of PQQ in mammalian fluids, if any, exists as PQQ derivatives, most likely as type 1 oxazole derivative.

REFERENCES

- (1) J. A. Duine, J. Jr. Frank, and J. K. van Zeeland, *FEBS. Lett.*, 108 (1979) 443.
- (2) S. A. Salisbury, H. S. Forret, W. B. T. Cruse, and O. Kennard, *Nature*, 280 (1979) 843.
- (3) C. L. Lobenstein-Verbeek, J. A. Jongejan, J. Frank, and J. A. Duine, *FEBS Lett.*, 170 (1984) 305.
- (4) M. Ameyama, M. Hayashi, K. Matsushita, E. Shinagawa, and O. Adachi, *Agric. Biol. Chem.*, 48 (1984) 561.
- (5) W. S. McIntire and J. T. Stults, *Biochem. Biophys. Res. Commun.*, 141 (1986) 562.
- (6) F. M. D. Vellieux and W. G. Hol, *FEBS Lett.*, 255 (1989) 460.
- (7) R. Flückiger, T. Woodtli, and P. M. Gallop, *Biochem. Biophys. Res. Commun.*, 153 (1988) 353.
- (8) J. A. Jongejan and J. A. Duine eds., *PQQ and Quinoproteins*, Kluwer Academic Publishers, Dordrecht, 1989, section: Analysis of PQQ.
- (9) S. M. Janes, D. Mu, D. Wemmer, A. J. Smith, S. Kaur, D. Maltby, A. J. Burlingame, and J. P. Klinman, *Science*, 248 (1990) 981.
- (10) D. E. Brown, M. A. McGuirl, D. M. Dooley, S. M. Janes, D. Mu, and J. P. Klinman, *J. Biol. Chem.*, 266 (1991) 4049.
- (11) W. S. McIntire, D. E. Wemmer, A. Chistoserdov, and M. E. Lidstrom, *Science*, 252 (1991) 817.
- (12) P. M. Gallop, M. A. Paz, R. Flückiger, and E. Henson, *Abstract of the 2nd International Symposium on PQQ and Quinoproteins, Nov 1991, Yamaguchi, Japan, 1991*, p. 40.
- (13) J. Killgore, C. Smidt, L. Duich, N. Romero-Chapman, D. Tinker, K. Reiser, M. Merko, D. Hyde, and R. B. Rucker, *Science*, **245** (1989) 850.
- (14) M. Ameyama, K. Matsushita, E. Shinagawa, and O. Adachi, *Vitam. Horm.*, 46 (1991) 229.
- (15) H. Nishigori, *Nippon Nogeikagaku Kaishi*, 66 (1992) 1657; and references cited therein.

- (16) Y. Esaka, K. Kano, M. Sukeguchi, and M. Goto, *Anal. Sci.*, 9 (1993) 207.
- (17) J. A. Duine, J. Frank, Jzn., and J. A. Jongejan, in A. Meister (Editor), *Advances in Enzymology*, vol. 59, John Wiley & Sons, New York, 1987. pp. 170-212; and references cited therein.
- (18) J. A. Jongejan and J. A. Duine eds., *PQQ and Quinoproteins*, Kluwer Academic Publishers, Dordrecht, 1989, section: Reaction of PQQ.
- (19) S. Itoh and Y. Ohshiro, *Bioorg. Chem.*, 19 (1991) 169; and references cited therein.
- (20) D. R. Sleath, J. B. Noar, G. A. Eberlein, and T. C. Burice, *J. Am. Chem. Soc.*, 107 (1985) 3328.
- (21) M. A. G. van Kleef, J. A. Jongejan, and J. A. Duine, *Eur. J. Biochem.*, 183 (1989) 41.
- (22) Itoh, M. Mure, A. Suzuki, H. Murao, and Y. Ohshiro, *J. Chem. Soc. Perkin Trans. 2*, (1992) 1245.
- (23) N. J. Reinhoud, U. R. Tjaden, H. Irth and J. Vandergreef, *J. Chromatogr.*, 574 (1992) 327.
- (24) K. Kano, K. Mori, B. Uno, T. Kubota, T. Ikeda, and M. Senda, *Bioelectrochem. Bioenerg.*, 23 (1990) 227.
- (25) K. Kano, K. Mori, B. Uno, T. Kubota, T. Ikeda, and M. Senda, *Bioelectrochem. Bioenerg.*, 24 (1990) 193.
- (26) J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.*, 53 (1981) 1298.
- (27) C. Schwer and E. Kennder, *Anal. Chem.*, 63 (1991) 1801.
- (28) O. Adachi, Nippon Nogeikagaku Kaishi, 66 (1992) 1665; and references cited therein.
- (29) S. Itoh, N. Kato, M. Mure, and Y. Ohshiro, *Bull. Chem. Soc. Jpn.*, 60 (1987) 420.
- (30) M. Mure, S. Itoh, and Y. Ohshiro, *Chem. Lett.*, (1989) 1491.
- (31) M. Mure, K. Nii, S. Itoh, and Y. Ohshiro, *Bull. Chem. Soc. Jpn.*, 63 (1990) 417.

3.3 Kinetic Analysis of Oxazolopyrroloquinoline Formation in the Reaction of Coenzyme PQQ with Amino Acids by Capillary Zone Electrophoresis

To date, the existence of three quinonoid redox cofactors, pyrroloquinoline quinone (PQQ), topa quinone (TPQ) and tryptophan tryptophyl quinone (TTQ), has been confirmed. TPQ is an amino acid-derived cofactor associated tightly with bovine plasma amine oxidase matrix through an amide linkage¹ and has been reported to be distributed in nature, not only in mammals.^{2,3} TTQ is also an amino acid-derived cofactor, which is covalently bound to methylamine dehydrogenase from a soil bacteria.⁴ On the other hand, PQQ has been confirmed as a non covalently bound redox cofactor of several bacteria.^{5,6} Therefore free PQQ is proposed to exist in mammalian bodies also as a "vitamin-like" compound,⁷ although the occurrence of PQQ as a cofactor of mammalian enzymes has not been proved yet. On the other hand, much attention has been attracted by several pharmaceutical activities of PQQ, for example, a therapeutic effect on cataract⁸⁻¹² and a stimulative effect to produce nerve growth factor (NGF) which is considered as a potent-drug for Alzheimer's syndrome.¹³ From these viewpoints, reliable detection techniques of PQQ and its related compounds in biological media have been required in various fields. However, the detection of free PQQ in mammalian fluids is very difficult because of its extremely low concentration, if any, and its high reactivities toward nucleophilic reagents such as amino acids.^{14,15} Our previous work has shown that most of PQQ spiked in bovine serum is converted immediately into the unsubstituted OPQ (OPQ1).^{16,17} Therefore, study of the formation of OPQs as derivatives of PQQ is very important in considering the occurrence and/or role of PQQ in mammals.

In our previous paper, we have emphasized the special feature of capillary zone electrophoresis (CZE) as a tool for separation analysis of PQQ and OPQs.¹⁷ Our aim in this work is to discuss the OPQ formation in biological media from the kinetic points of view with the aid of CZE.

EXPERIMENTAL

Materials

Pyrroloquinoline quinone (PQQ) was obtained from Ube Industry (Tokyo, Japan) and used as received. All other chemicals used were of analytical grade.

Apparatus

Electrophoretic separations were performed in a fused silica tubing (GL Science, Tokyo, Japan) with 0.05-mm i.d. and a column length of 600 mm with a separation length of 350 mm unless noted otherwise. All other details in CZE was the same as those described in a previous paper.¹⁷

The generation of the reduced PQQ was monitored by rotating disk amperometry using a Yanagimoto P-1000 potentiostat, a Nikko Keisoku SC-5 motor-speed controller and a Nikko Keisoku RRDE-1 rotating disk motor. A gold disk electrode with a diameter of 2 mm was used as a working electrode. The electrode was set at potentials sufficiently positive than the redox potential of PQQ.^{18,19}

Sample preparation

PQQ-spiked serum sample was pretreated for CZE analysis as reported in our previous paper.¹⁷ The pretreatment of the sample was performed 48 h after the PQQ-spiking.

Reaction solutions of amino acids and PQQ were prepared as follows. A $5.0 \times 10^{-5} \text{ dm}^3$ of PQQ solution ($2.92 \times 10^{-3} \text{ mol dm}^{-3}$) was added to $5.0 \times 10^{-4} \text{ dm}^3$ of each of buffer solutions containing amino acid at certain concentrations. The buffer solutions were 0.05 mol dm^{-3} phosphate buffer (pH 3, 6.5, 7.4) and 0.1 mol dm^{-3} borate buffer (pH 9.0). Incubations were performed at room temperature. These reaction solutions were sampled periodically and subjected to CZE analysis without any pretreatments. For quantitative analysis, *o*-nitrobenzoic acid was used as an internal standard. The detection wavelength was set at 249 nm.

The reduction of PQQ with glycine was carried out under anaerobic conditions. An aliquot of PQQ solution was syringed into $5.0 \times 10^{-2} \text{ mol dm}^{-3}$ glycine solution to start the reaction (final concentration: $7.0 \times 10^{-6} \text{ mol dm}^{-3}$). The buffer solutions used were made from 0.1 mol dm^{-3} phosphate ($6.5 < \text{pH} < 8.5$), 0.2 mol dm^{-3} tris(hydroxymethyl)-aminomethane ($8 < \text{pH} < 9$), 0.1 mol dm^{-3} carbonate ($9 < \text{pH} < 11$), and 0.05 mol dm^{-3} phosphate ($\text{pH} > 11$).

RESULTS AND DISCUSSION

Products Analysis in PQQ-spiked Bovine Serum

There exist many kinds of nucleophilic substances in serum at high concentrations. Therefore, PQQ can be converted into several derivatives, because of its high reactivity toward nucleophiles. Although only OPQ1 was detected in a PQQ-spiked ($5.0 \times 10^{-6} \text{ mol dm}^{-3}$) serum sample as described in our previous paper,¹⁷ several other kinds of undetectable derivatives could be generated in trace amounts. In this work, the concentration of PQQ spiked in serum was increased up to $1.0 \times 10^{-4} \text{ mol dm}^{-3}$ and a 67-fold concentration was carried out in the sample preparation. Figure 1 shows an electropherogram of the PQQ-spiked sample detected at 420 nm, in which the capillary length was 700 mm (separation length was 550 mm) and the electrolyte solution was 0.01 mol dm^{-3} phosphate buffer (pH 7.5) containing 5% (v/v) dimethyl sulfoxide (DMSO).

The addition of DMSO was essential for good reproducibility to avoid adsorbing OPQs on the inner wall of the capillary.¹⁶ More than 90% of the spiked PQQ was converted into OPQ1. In addition to the main peak of OPQ1 and the peak of the unreacted PQQ (3.7 % compared with OPQ1), at least four minor peaks (numbered from 1 to 4) are observed at about 0.3 -0.4 % each compared with OPQ1. The four peaks were assigned, respectively, to OPQ2s derived from tyrosine, phenylalanine, leucine (or *iso*-leucine) and valine by the addition of the corresponding standard OPQs. Several other minor products were also detected at

249 nm (not shown). Judging from the peak height ratio at 249 and 420 nm,^{15,17,21} they would not be OPQ. The predominant formation of OPQ1 compared with OPQ2s seems to be governed mainly by kinetics of the OPQ formation.

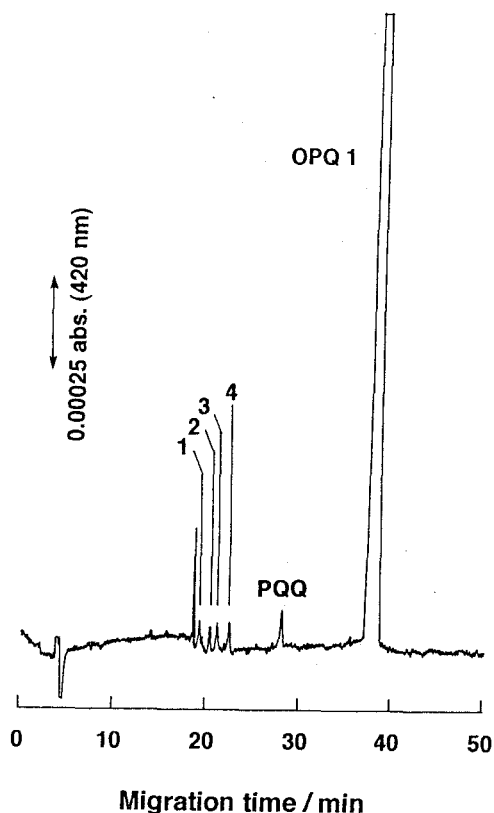


Figure 1 Electropherogram of PQQ-spiked (1.0×10^{-4} mol dm⁻³) bovine serum sample.

Electrolyte solution, 0.01 mol dm⁻³ phosphate buffer (pH 7.5) containing 5 % DMSO; capillary, 700 mm \times 0.05 mm i.d. (550 mm effective length); separation voltage, 20 kV, current, 10 μ A; injection (siphoning), 15 cm. 7s; detection wavelength 420 nm; Peaks 1-4 can be assigned to OPQ2 from Tyr, Phe, Leu (or Ile) and Val, in turn.

Kinetics of OPQ formation

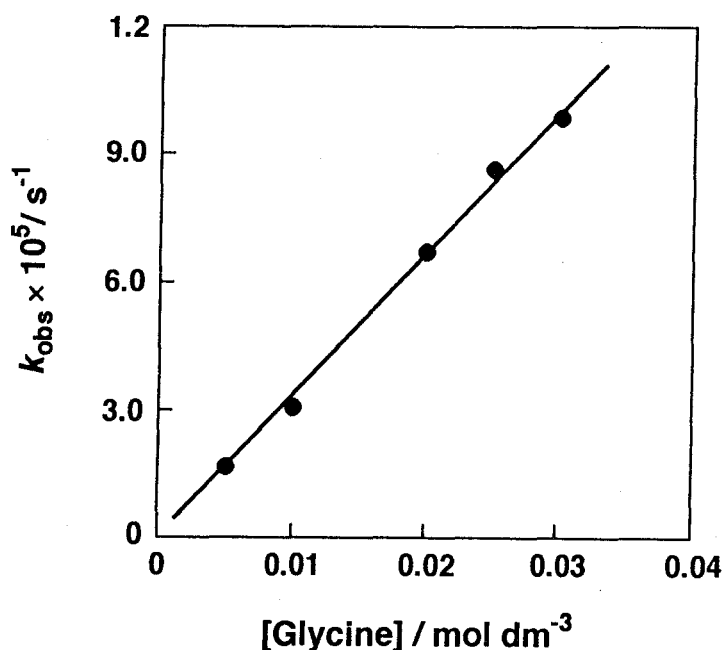


Figure 2 Dependence of k_{obs} on the concentration of glycine at pH 6.5.

The reactions of PQQ with amino acids were carried out at room temperature under aerobic conditions. The time course of the PQQ disappearance and/or the OPQ generation was measured by CZE. In the presence of glycine, PQQ was converted exclusively into OPQ1. The OPQ1 formation proceeded in pseudo-first order in the concentration of PQQ at pH 6.5 in the presence of excess glycine ($[\text{glycine}] = 5.0 \times 10^{-3} - 3.0 \times 10^{-2} \text{ mol dm}^{-3} \gg [\text{PQQ}]_0 = 2.92 \times 10^{-4} \text{ mol dm}^{-3}$). As shown in Fig. 2, a plot of the observed pseudo-first order rate constant of the OPQ formation (k_{obs}) vs. $[\text{glycine}]$ gave a linear relationship. This result indicates that the reaction proceeds in first order in each of the concentrations of PQQ and glycine. Valine gave the corresponding OPQ2 (Val-OPQ2) exclusively. Serine gave OPQ1 at low pH, while it gave the corresponding OPQ2 (Ser-OPQ2) at high pH, predominantly. The OPQ formation from valine and serine also proceeded in first order in each of the concentration of PQQ and the amino acids.

Table 1 Second-order Rate Constants for OPQ generation^{a)}

		pH 3.0	pH 6.5	pH 7.4	pH 9.0
OPQ1	glycine	2.2×10^{-3}	3.1×10^{-3}	4.2×10^{-3}	2.3×10^{-2}
	serine	-3.3×10^{-4}	1.4×10^{-3}	9.2×10^{-3}	
OPQ2	serine	1.2×10^{-3}	2.2×10^{-4}	-	-
	valine	5.0×10^{-4}	1.7×10^{-4}	9.4×10^{-5}	2.5×10^{-5}

a) values in $\text{dm}^3 \text{mol}^{-1} \text{s}^{-1}$ at room temperature.

Table 1 summarizes the second order rate constants (k) of the OPQ formation with glycine, valine and serine at pH 3.0, 6.5, 7.4 and 9.0. The OPQ1 formation from glycine was increased with a rise in pH. The Val-OPQ2 formation was increased with decreasing pH. The formation of OPQ1 and OPQ2 from serine was increased under basic and acidic conditions, respectively. The rate constant of the OPQ1 formation from glycine is 45 times larger than that of the Val-OPQ2 formation at pH 7.4. The OPQ1 formation from serine is also faster than the Val-OPQ2 formation. These results suggest that the OPQ1 formation is inherently faster than the OPQ2 formation at biological pH, although the predominant (or exclusive) OPQ1 formation is restricted to glycine, tryptophan, threonine, serine, and tyrosine¹⁷. This kinetic consideration explains well the finding of the predominant OPQ1 formation from PQQ spiked in bovine serum.

Mechanism of OPQ formation

Besides the OPQ formation, it is well known that amino acids are oxidatively decarboxylated with PQQ to yield its quinol and aminophenol forms (Fig. 5-3).^{14,21-24} Under aerobic condition, the reduced PQQ is reoxidized to PQQ.¹⁵ Itoh et al. discussed the kinetics of the reduction of PQQ with glycine

under anaerobic conditions.²¹ The PQQ reduction proceeds in first order in [PQQ] and in both second and first order in [glycine] as expressed by eq 1.

$$d([\text{aminophenol}] + [\text{quinol}])/dt = [\text{PQQ}](k_1[\text{glycine}] + k_2[\text{glycine}]^2) \quad (1)$$

The terms involving the second and first order dependence on [glycine] correspond to the quinol and the aminophenol formation, respectively (Fig. 3). Glycine works as a catalyst as well as a substrate in the quinol formation process.²¹

The OPQs are considered to be generated in side reactions of the catalytic oxidation of amino acids with PQQ.^{15,21,22,25} Since the OPQ formation is irreversible and then OPQs are accumulated as final products during the redox cycling under aerobic conditions. As described above, the OPQ formation proceeds in first order in the concentration of amino acids. This result strongly suggests that OPQs are produced in by-path reactions of the redox cycling reaction via the aminophenol as shown in Fig. 3. The reaction between PQQ and amino acids proceeds via an ionic mechanism involving carbinolamine-type intermediate which is dehydrated to give iminoquinone.²¹ When C $_{\alpha}$ -C $_{\beta}$ bond cleavage followed by decarboxylation from the iminoquinone intermediate, OPQ1 is generated (Path A). In contrast, when decarboxylation is facilitated in the iminoquinone, OPQ2 as well as aminophenol is produced via the corresponding imine (Path B). The latter pathway is a major one for most of amino acids as reported in our previous paper.¹⁷

In the case of glycine, OPQ1 can be produced via Path B as well as Path A of Fig. 3. Considering the proposal that the OPQ formation is a by-path of the redox cycling, we studied the kinetics of the PQQ reduction with glycine under anaerobic and pseudo-first order conditions using rotating disk amperometry. In this experiment, aminophenol and quinol are simultaneously detected.²⁶ Figure 4 shows a pH dependence of the pseudo-first order rate constant (k^*_{obs}). The pseudo-first order rate constants were evaluated from the current-time curves, in which the oxidation current was converted into the concentration of the reduced PQQ using Levich equation.²⁰ The remarkable increase in k^*_{obs} up to pH 9 is ascribed to an increase in the fraction of the conjugate base of glycine (^-OOC -

CH₂NH₂, $pK_a = 9.6$), which is a true nucleophilic substrate. The decrease in k^*_{obs} over pH 10 is attributable to a decrease in the fraction of the redox active PQQ as an true oxidant due to the hydration (pseudo $pK_a = 10.3$).^{18,25} The pH dependence of the OPQ1 formation from glycine resembles that of the PQQ reduction qualitatively. This supports the proposal concerning the by-path mechanism. The fact that the OPQ1 formation from glycine is much faster compared with serine at high pH is also explained by our kinetic result that k^*_{obs} of glycine is larger than that of serine (ca. 5 times larger at pH 9).

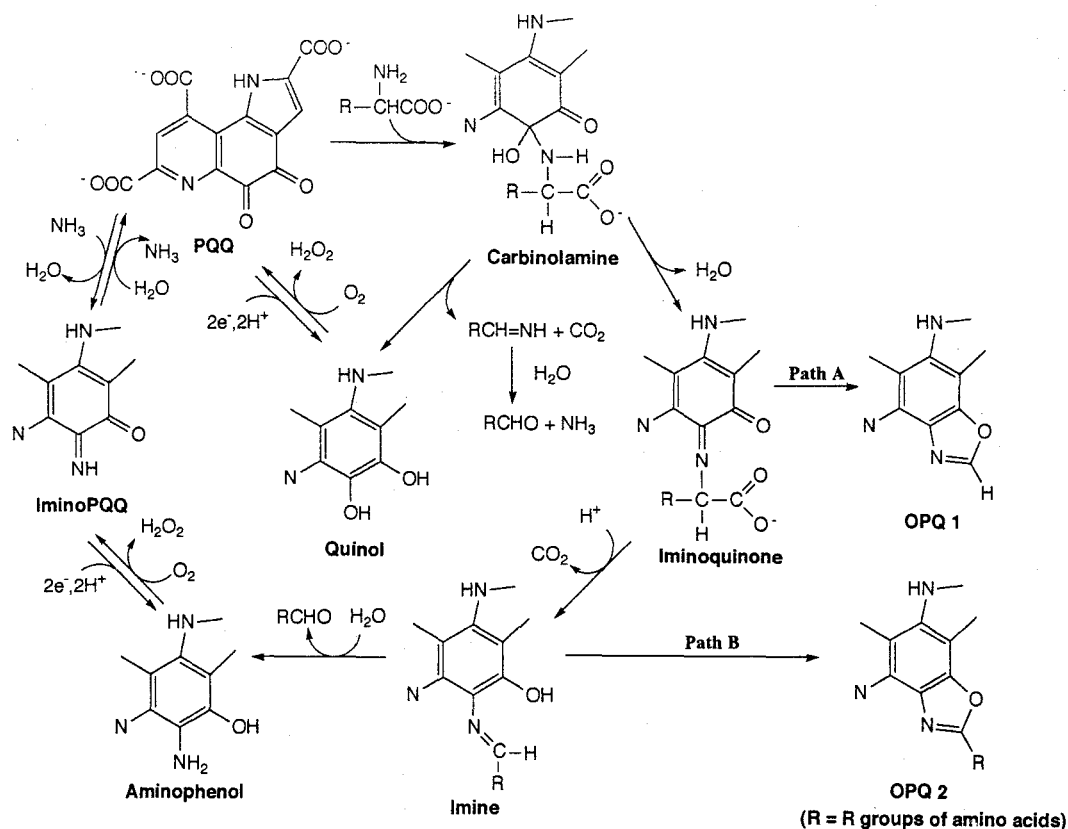


Figure 3 Catalytic oxidation of amino acids with PQQ and the OPQ formation.

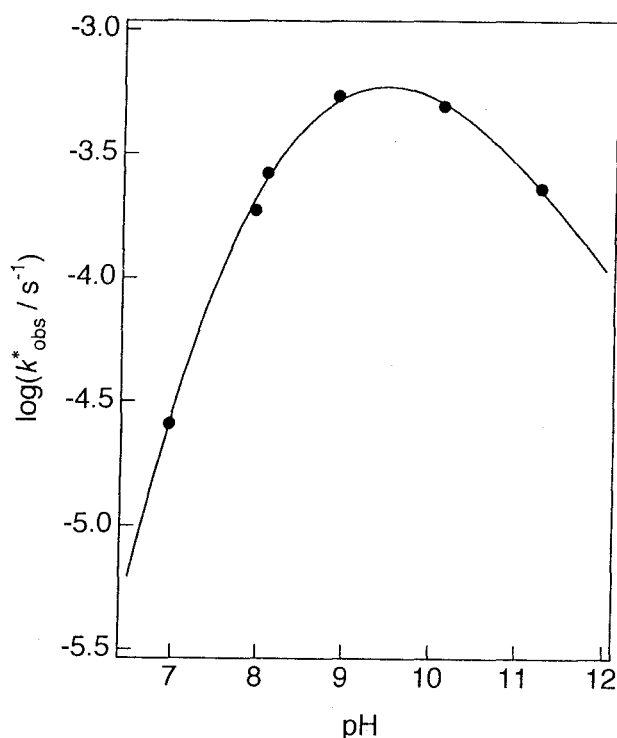


Figure 4 pH Dependence of k^*_{obs} under anaerobic conditions.

Duine et al. have revealed that the OPQ formation involves oxygen consumption.¹⁵ Taking their finding into account, we propose detailed reaction pathways to generate OPQ1 and OPQ2, as shown in Fig. 5. The formation of Val-OPQ2 and Ser-OPQ2 was enhanced with decreasing pH. This suggests the involvement of an acid-catalysis. At lower pH, the imine intermediate in the redox cycle will be protonated to give the corresponding imino cation, which will suffer a ring closure by nucleophilic attack of phenolic oxygen. The resultant intermediate will be oxidized to give OPQ2 (Fig. 5, Path B).

For tyrosine and serine, the predominant product is switched from the corresponding OPQ2 to OPQ1 with increasing pH. As in the case of Val-OPQ2, the OPQ2 formation from these two amino acids is slowed down with increasing pH. On the other hand, the OPQ1 formation will be enhanced by the base-attack on tyrosine or serine residue to cause C α -C β cleavages in the corresponding iminoquinone intermediate (Fig. 5, Path A). The imine intermediate will be oxidized by oxygen, as in the case of di- or polyphenols. The resultant iminoquinone will suffer ring closure by nucleophilic attack of the carbonyl group. After then decarboxylation will follow to yield OPQ1. Thus Path A overcomes Path B at elevated pH. Threonine and tryptophan generate OPQ1 predominantly in the broad pH range.¹⁷ The base-catalytic C α -C β cleavage model may not be applicable to these amino acids. Some structural factors might also govern the reaction pathway.

As mentioned in the beginning, PQQ possesses biological and/or pharmacological activities. For example, addition of PQQ *in vitro* promotes the NGF production over 40 times in an NGF productive cell line.¹³ However, the promotive effect was scarcely observed in the case of administration of PQQ *in vivo*.¹³ This conflicting result may be explained by the finding that PQQ in serum is converted into several inactive species, especially OPQ1. For further discussion, detailed speciation of PQQ administrated into mammalian fluids will be required to identify a true species having biological activity. On the other hand, it is interesting that Ser-OPQ2 exhibits much effective growth stimulating activity for bacteria, compared with PQQ itself.²⁷ As described here, the *in vivo* generation of Ser-OPQ2 was not observed at least in bovine serum. Therefore, it seems to be important that PQQ is derivatized into much stable forms before administration. Such derivatization will be also useful in the identification of a biologically active form of PQQ in mammals.

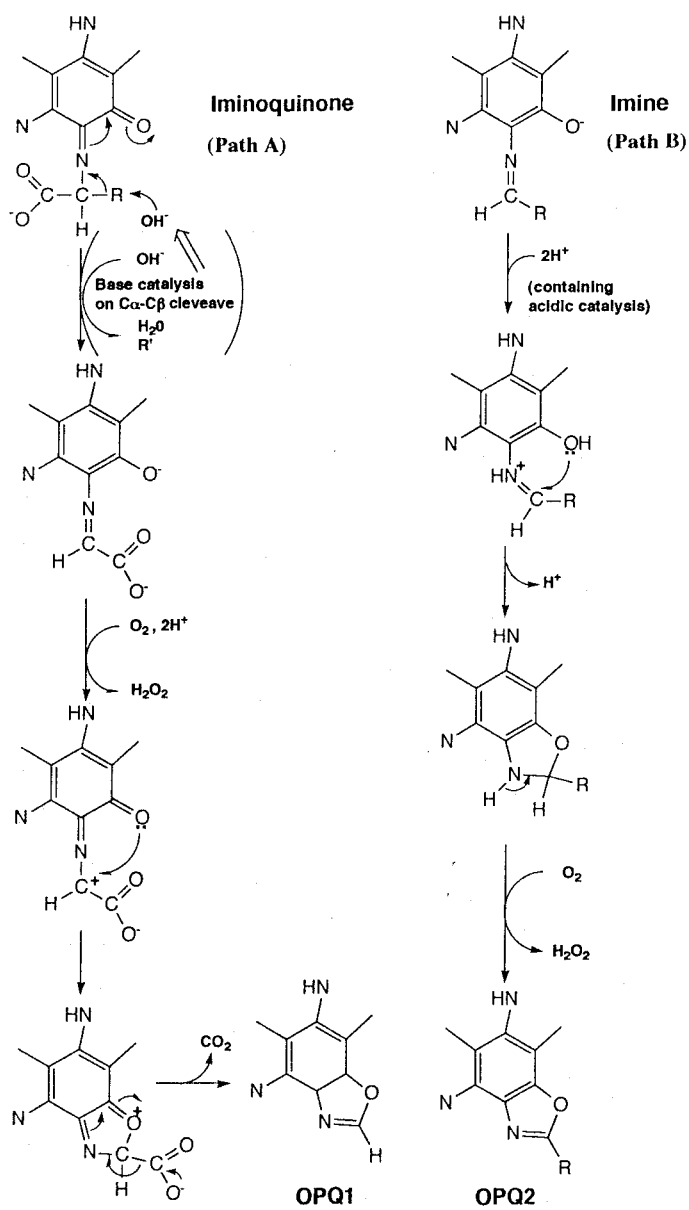


Figure 5 Proposed mechanism of the formation of OPQ1 and OPQ2.

SUMMARY

Kinetics of the formation of oxazolopyrroloquinoline derivatives (OPQ) from coenzyme pyrroloquinoline quinone (PQQ) and three α -amino acids (glycine, serine and valine) was studied with the aid of capillary zone electrophoresis. Glycine and valine were, respectively, converted exclusively into the unsubstituted OPQ (OPQ1) and an OPQ with the valinyl residue (Val-OPQ2), while serine gave OPQ1 and Ser-OPQ2 under basic and acidic conditions, respectively. The OPQ formation exhibited a first order dependence on each of PQQ and the amino acids. The OPQ1 generation from glycine is 45 times faster than that of Val-OPQ2 at pH 7.4. This result is in accord with an observation that PQQ spiked in bovine serum was converted predominantly into OPQ1. The mechanism of the OPQ formation is discussed in detail.

REFERENCES

- (1) S. M. Janes, D. Mu, D. E. Wemmer, A. J. Smith, S. Kaur, D. Maltby, A. L. Burlingame and J. P. Klinman, *Science*, 248 (1990) 981.
- (2) J. van Iersel, R. A. van der Meer and J. A. Duine, *Eur. J. Biochem.*, 161 (1986) 415.
- (3) D. Mu, S. M. Janes, A. J. Smith, D. E. Brown, D. M. Dooley and J. P. Klinman, *J. Biol. Chem.*, 267 (1992) 7979.
- (4) W. S. McIntire, D. E. Wemmer, A. Chistoserdov and M. E. Lidstrom, *Science*, 252 (1991) 817.
- (5) S. A. Salisbury, H. S. Forrest, W. B. T. Cruse and O. Kennard, *Nature*, 280 (1979) 843.
- (6) J. A. Duine, J. Frank and J. K. van Zeeland, *FEBS. Lett.*, 108 (1979) 443.
- (7) J. Killgore, C. Smidt, L. Duich, N. Romero-Chapman, D. Tinker, K. Reiser, M. Melko, D. Hyde and R. B. Rucker, *Science*, 245 (1989) 850.
- (8) H. Nishigori, M. Yasunaga, M. Mizumura, J. W. Lee and M. Iwatsu, *Life Sci.*, 45 (1989) 593.
- (9) H. Nishigori, *Nippon Nogeikagaku Kaishi*, 66 (1992) 1657.
- (10) H. Nishigori, *Kagaku To Seibutsu*, 30 (1992) 78.
- (11) T. Urakami, *Nippon Nogeikagaku Kaishi*, 65 (1991) 380.
- (12) T. Urakami, M. Oda, C. Itoh, H. Kobayashi, T. Nagai and K. Sugawara, EP 429,333 /1991 (*Chem. Abstr.*, 116, 5, 39769w).
- (13) K. Yamaguchi, A. Sasano, T. Urakami, T. Tsuji and K. Kondo, *Biosci. Biotech. Biochem.*, 57 (7) (1993) 1231.
- (14) J. A. Duine, J. Frank and J. A. Jongejan, in *Advnces in Enzymology*, ed. A. Meister, John Wiley & Sons, New York, 1987, vol. 59, p. 169.
- (15) M. A. G. van Kleef, J. A. Jongejan and J. A. Duine, *Eur. J. Biochem.*, 183 (1989) 41.
- (16) Y. Esaka, K. Kano, M. Sukeguchi and M. Goto, *Anal. Sci.*, 9 (1993) 207.
- (17) Y. Esaka, Y. Yamaguchi, K. Kano and M Goto, *J. Chromatogr. A*, 652 (1993) 225.

- (18) K. Kano, K. Mori, B. Uno, T. Kubota, T. Ikeda and M. Senda, *Bioelectrochem. Bioenerg.*, 23 (1990) 227.
- (19) K. Kano, K. Mori, B. Uno, T. Kubota, T. Ikeda and M. Senda, *Bioelectrochem. Bioenerg.*, 24 (1990) 193.
- (20) A. J. Bard and L. R. Faulkner, *Electrochemical Methods*, John Wiley & Sons, New York, 1980, Chap. 8.
- (21) S. Itoh, M. Mure, A. Suzuki, H. Murao and Y. Ohshiro, *J. Chem. Soc. Perkin Trans. 2*, 1992, 1245.
- (22) Y. Ohshiro and S. Itoh, *Bioorg. Chem.*, 19 (1991) 169.
- (23) S. Itoh, N. Kato, Y. Ohshiro and T. Agawa, *Tetrahedron Lett.*, 25 (1984) 4753.
- (24) R. Flückiger, T. Woodtli and P. M. Gallop, *Biochem. Biophys. Res. Commun.*, 153 (1988) 353.
- (25) K. Kano, K. Mori, B. Uno and M. Goto, *J. Electroanal. Chem.*, 293 (1990) 177.
- (26) D. R. Sleath, J. B. Noar, G. A. Eberlein and T. C. Burice, *J. Am. Chem. Soc.*, 107 (1985) 3327.
- (27) O. Adachi, *Nippon Nogeikagaku Kaishi*, 66 (1992) 1665.

CHAPTER 4

ON-LINE HPLC DETERMINATION OF ENZYMATIC ACTIVITY OF ALKALINE PHOSPHATASE IN NATURAL WATER

Alkaline phosphatase (APase) is contained in various kind of organism such as human, animal, fish, bacteria etc.¹ and also in natural waters where such organisms are living.² It is further known that its enzyme detection or the chromatographic (or electrophoretic) pattern, as well as APase amounts, is useful for medical diagnosis of the animal organs.³ In the case of natural water, the analysis of APase gives the interesting understandings for physicochemical behaviors in nature; for instance, the speciation for zinc in natural water^{2,4} or the ecological mechanism of phosphorus cycles.⁵

In order to analyze APase in biological or environmental samples, the separation from other components is first required. In these studies HPLC (high performance liquid chromatography) has been generally employed, where two detecting principles of APase have been utilized. One is the method based on the UV-absorption of APase, and the other is that utilizing its enzymatic activity. From the viewpoint of the sensitivity as well as the selectivity, the latter is much superior to the former. In this paper, fundamental studies on the on-line HPLC detection of enzymatic activity of alkaline phosphatase (APase) will be described.

EXPERIMENTAL

Materials

Escherichia Coli alkaline phosphatase (APase, Product No.4151, enzyme activity = 31 U/mg) and 4-methylumbelliferyl phosphate (4-MUP) and 4-methylumbelliferone (4-MU) were purchased from Sigma (St. Louis, USA). Disodium *p*-nitrophenylphosphate (*p*-NPP) and *p*-nitrophenol (*p*-NP) were obtained from Katayama Chemical (Osaka, Japan). All other chemicals were of analytical grade.

Apparatus

The block diagram of the experimental system used is shown in Fig. 1.

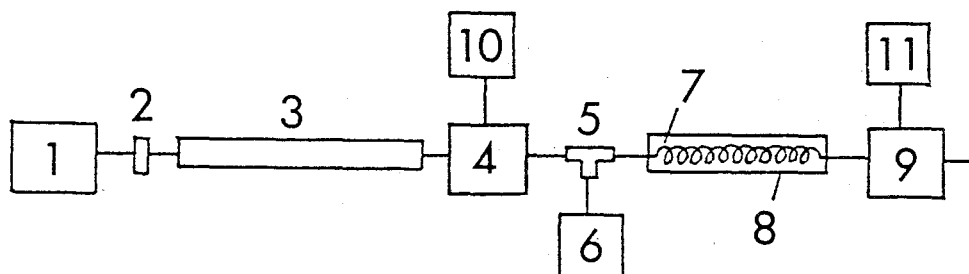


Figure 1 Block diagram of the on-line HPLC system used for analysis of APase in natural water.

1. pump (eluent), 2. loop injector, 3. column, 4. UV-vis detector, 5. T-tube, 6. pump (4-MUP), 7. reactor (0.5 mm × 50 m), 8. thermostat, 9. fluorescence detector, 10, 11. recorder.

The FPLC system (Pharmacia-LKB) with a Superose 12 column of 30 cm × 10 mm i.d., a MV-7 valve injector and a LCC-500 Plus controller were used for size exclusion chromatography (SEC). Tris-HCl buffer solution (0.1 M) of pH 8 was used as an eluent at a flow rate of 0.5 ml/min. After SEC separation, the chromatograms were monitored at the 2 stages; first by a UV-monitor (LKB model 2124) at 280 nm to detect various kinds of organic compounds contained in natural water and then by a fluorometric one (JASCO model 812-FP) to detect APase activity using a 4-MUP substrate. The substrate was dissolved in an eluting buffer

to be a 0.1 mM solution and added to column effluent through a T-tube at a flow rate of 0.1 ml/min. As a reaction tube the PTFE tubing of 0.5 mm i.d. \times 50 m length was used, which was installed in thermostat, although all chromatographic experiments were performed at room temperature. The fluorescence from 4-MU as the reaction product of 4-MUP was monitored at 362 and 442 nm as excitation and emission wavelengths, respectively.

Sample preparation

Pond water samples were filtered through a 0.45 mm membrane filter (Advantec), and concentrated by 160-fold with an ultrafiltration system (Advantec) using a 10,000-M.W. wet-type ultrafilter (43 mm i.d.) equipped in a UHP-43K stirred cell (70 ml volume), which was connected to a 1 liter volume RP-1 reservoir.

RESULTS AND DISCUSSION

Comparison of detection limits of alkaline phosphatase (APase) by the methods based on fluorometry and spectrophotometry in a batch method

As the detection method of the enzymatic activity of APase, two methods have been reported. One is based on spectrophotometry⁵ and the other on fluorometry.⁶ In the present experiment, these two methods were examined to compare their sensitivities, using p-NPP and 4-MUP as the substance.

These substrates were dissolved in 0.6 M ($M = \text{mol/dm}^3$) Tris-HCl solution (pH 8.0) to be 0.1 mM. The results obtained by a batch method *E. Coli* APase are shown in Table 1.

Table 1 Comparison of the detection limits for APase by spectrophotometry and fluorometry in a batch method.

Reaction time ^{a)}	Detection limit / ppb	
	Spectrophotometry (p-NPP) ^{b)}	Fluorometry (4-MUP) ^{b)}
3 min	1100	5.3
1 h	55	0.26
24 h	2.3	0.011

a) Incubation time. b) Substrate used.

The detection limits in Table 1 were estimated as follows. In the case of spectrofluorometry, the detection limit was calculated as the amount of APase which gave the signal corresponding to twice the standard deviation of the blank signal, while in the case of spectrophotometry it was done as the amount to give the signal corresponding to $S/N=2$ (S; absorption signal intensity in absorbance, N; baseline noise of the blank signal). It can be seen from Table 1 that the spectrofluorometric detection gave better sensitivity by about 200-times than the spectrophotometric one.

On-line combination of the HPLC separation of APase and the fluorometric detection of its enzymatic activity

The experiment was then extended to the on-line post-column HPLC detection. The experimental system used was similar to that shown in Fig. 1, except for the use of 0.02 mM *p*-NPP substrate dissolved in 0.1 M Tris-HCl solution as an eluting buffer and a shorter reaction tube of 7 m length (0.5 mm i.d.). In both cases of fluorometric and photometric detections the detection limits were estimated according to $S/N=2$. The detection limits were calculated to be

about 57 and 620 pg for the on-line fluorometric and photometric detections, respectively. Thus, in the on-line detection the fluorometric method gave better sensitivity by about 10-times than the photometric one, although 200-times of sensitivity difference was obtained in the batch method, as described above. Such poorer sensitivity in the on-line fluorometric detection was mainly caused by the less stable baseline.

Using a longer reaction tube of 50 m, the fluorometric measurement of enzymatic activity with this detection system gave better sensitivity by about 700-times than the "direct" UV-detection of APase.

Determination of APase activity in natural water

The analytical method with the above system for the on-line fluorometric detection of APase activity developed in the present experiment was applied to the analysis of natural water sample. Samples were collected from the Kagamigaike pond in the campus of Nagoya University. The chromatograms of a pond water sample concentrated by 160-fold are shown in Fig. 2, where the solid and broken lines were the chromatograms observed by an UV absorption and a fluorometric detection of the enzymatic activity. Although it was not clear in the UV-monitored chromatogram, the fluorometric detection indicated existence of APase with similar M.W. to that of *E.Coli* APase, clearly.

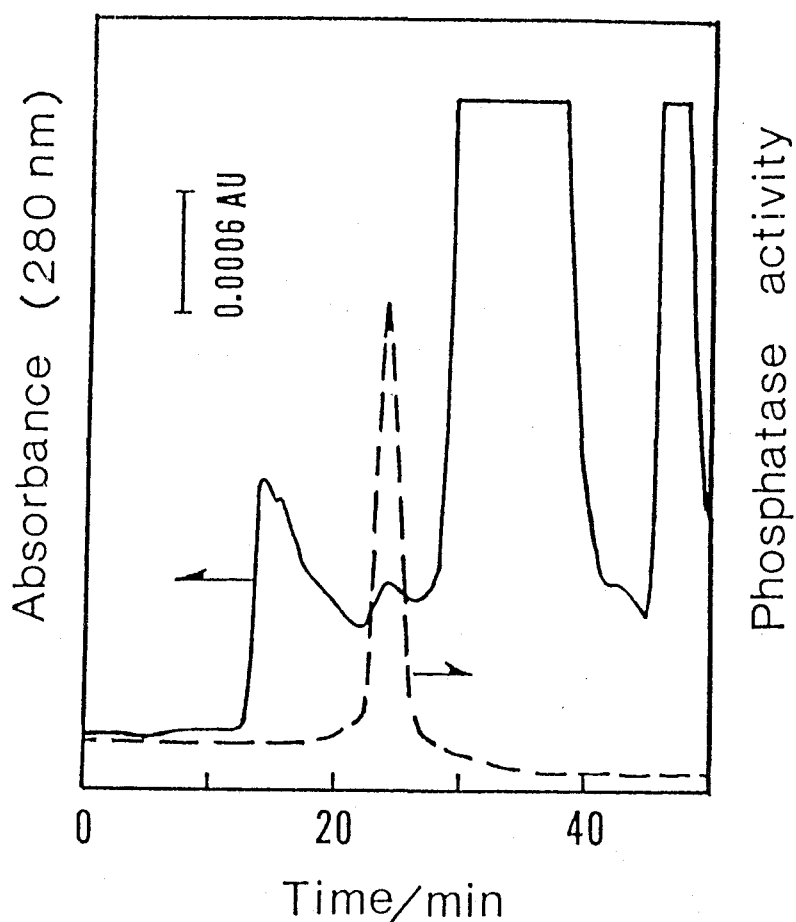


Figure 2 SEC chromatograms of for 160-fold concentrated pond water.

—: Spectrometric detection at 280 nm

---: Fluorometric detection of enzymatic activity

Injection volume of sample: 1 ml

SUMMARY

Fundamental studies on the on-line HPLC detection of enzymatic activity of alkaline phosphatase (APase) has been examined, where 4-methylumbelliferyl phosphate was used as a substrate. The technique developed was applied to detection of APase in natural water.

REFERENCES

- (1) W. Mayer-Sabellek, P. Shinha, and E. Kottgen, *J. Chromatogr.* 429 (1988) 419.
- (2) K. Kobayashi and H. Haraguchi, *Nippon Kaisui Gakkaishi*, 42 (1988) 155.
- (3) T. Komoda, I. Koyama, and Y. Sakagishi, *Tanpakushitu Kakusan Kouso*, 30 (1985) 1408.
- (4) K. Kobayashi, K. Fujiwara, and H. Haraguchi, *Kaiyo Kagaku*, 147 (1982) 267.
- (5) K. Kobayashi, K. Iwase, S. Hashimoto, H. Ueda, K. Fujiwara, A. Otuki, K. Fuwa, and H. Haraguchi, *Bull. Chem. Soc. Jpn.*, 60 (1987) 925.
- (6) S. Hashimoto, K. Kobayashi, K. Fujiwara, H. Haraguchi, and K. Fuwa, *Bunseki Kagaku*, 32 (1983) E177.

CONCLUSIONS

The results in the studies described in this thesis can be concluded as follows:

CHAPTER 1

It have been demonstrated that improved separation can be achieved by using agents with polyether structure as a matrix in CZE and as surfactants in MEKC. The present study has clearly demonstrated that the hydrogen-bonding interaction with the agents plays an important role. Hydrogen bond is a weak interaction, especially in aqueous media, but it can be concluded that the interaction is enough to affect the relative mobility of analytes in capillary electrophoresis.

Use of PEG as a matrix is very useful in CZE separation. The present NMR experiments have directly demonstrated that PEG works as a hydrogen acceptor via its polyether segments and in part as a hydrogen donor via its end hydroxyl groups. The temperature effect and the urea effect can be described fundamentally in terms of the hydrogen-bonding interaction between PEG and analytes. The hydrogen-bonding interaction in aqueous phase will not be so strong as demonstrated by NMR in CDCl_3 . However, the strength is fortunately appropriate for improving separation in CZE. Thus the CE method using PEG as a matrix would open routes to develop *electrostatic capillary electrophoresis*. From a physicochemical point of view, this method might be applicable to an estimation of the hydrogen-donating property of analytes or hopefully of the hydrogen-accepting property of analytes using some hydrogen-donating matrix.

The present study suggested the occurrence of some other minor interactions in addition to the hydrogen-bonding interaction, such as the hydrophobic interaction and the steric interaction. These interactions work in conjunction for hydrogen-donating analytes and sometimes effectively for hydrogen-donating inactive analytes to improve the separation.

On the other hands, the incorporation of non-ionic surfactants with polyether moieties into SDS system to form the mixed micelle is found to provide a new pseudo-stationary phase in MEKC. Hydrogen-bonding interaction between analytes and the polyether moieties appears to work additively with the hydrophobic

interaction in the mixed micellar EKC, resulting in the improvement of the separation. The hydrogen-bonding interaction in MEKC is expected to be more effective than in CZE mode, because the hydrogen-bonding will form more strongly in the hydrophobic micellar core.

CHAPTER 2

When a standard sample of sperm whale myoglobin was separated by chromatofocusing, one main large peak and at least 8 small peaks were observed in the chromatogram. According to the present experimental results obtained from separation by size exclusion chromatography, the measurement of absorption spectra and estimation of pI values, it has been concluded that many isoforms exist in sperm whale myoglobin. Thus it is expected that chromatofocusing is a useful technique to obtain highly purified proteins of interest and to examine the heterogeneity of the proteins.

CHAPTER 3

The proposed electrochemical method is very useful to quantify compounds possessing PQQ-like catalytic activity toward glycine. The present research is suggestive of nucleophilic attack to PQQ in some biological fluids. For detail insight into the problem concerning the occurrence and/or the state of PQQ in mammalian, development of improved pretreatment and identification of the reaction products would be required.

Condensation products (OPQs) from the reaction of PQQ with several amino acids were successfully separated by CZE with organic modifiers. In this work, it was also appeared that CZE is very suitable for analysis of products from PQQ. The result in the products analysis of PQQ-spiked serum sample suggests that most of the PQQ in mammalian fluids, if any, exists as PQQ derivatives, probably as OPQ1.

CZE should be also a powerful tool for kinetic studies of OPQs formation. From the present kinetic studies and product analyses of the reaction of PQQ with amino acids, it has been revealed that PQQ is predominantly converted into OPQ1 during the redox cycling in mammalian fluids. Some OPQ2s are also generated.

However, the conversion factor is much lower than that of OPQ1 because of the larger kinetic barrier. The result is very important in pharmaceutical aspects. Administrated PQQ as the free form will be immediately and predominantly derived to OPQ1, which should be a pharmaceutically inactive form as reported in some cases. It suggests also necessity of derivatizations of PQQ to some active forms, for example, some OPQ2s, when PQQ is used as drugs.

CHAPTER 4

The present on-line detection method using HPLC is rapid and convenient for APase enzymatic activity analysis, and thus it may be useful for the studies on APase in natural waters as well as in biological samples. Identifying origin of APase in natural water will be valuable not only for biological interest but also for environmental interests. APases from mammals may use to indicate origin of water pollution.

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PUBLICATION LIST

1. Separation Characteristics of Whale Myoglobin by Chromatofocusing and Size Exclusion Chromatography with Aid of a Photodiode Array Detector
Yukihiro Esaka, Akio Hirose and Hiroki Haraguchi.
Anal. Sci., **7**(suppl.) (1991) 205-208.
2. Analysis of Myoglobin by Microcolumn Chromatography and Photodiode Array Detection.
Akio Hirose, Yukihiro Esaka and Hiroki Haraguchi
J. High Resol. Chromatogr., **14** (1991) 420-425.
3. On-line HPLC Detection of Enzymatic Activity of Alkaline Phosphatase in Natural Water Using Spectrofluorometric Detection.
Akio Hirose, Yukihiro Esaka, Michiru Ohta and Hiroki Haraguchi
Chem. Lett., **1993**, 307-310.
4. Electrochemical Detection of Pyrroloquinoline Quinone Coupled with Its Catalytic Function by Liquid Chromatography.
Yukihiro Esaka, Kenji Kano, Masatoshi Sukeguchi and Masashi Goto
Anal. Sci., **9** (1993) 207-211.
5. Separation of Amino Acid-oxazole Derivatives of the Redox Coenzyme Pyrroloquinoline Quinone by Capillary Zone Electrophoresis.
Yukihiro Esaka, Yasuo Yamaguchi, Kenji Kano and Masashi Goto
J. Chromatogr. A, **652** (1993) 225-232.
6. Electrochemical and Spectroscopic Characterization of Quinoid Cofactors.
Kenji Kano, Bunji Uno, Yukihiro Esaka, Toshio Mori and Masashi Goto
"Redox Mechanisms and Interfacial Properties of Molecules of Biological Importance", edited by F. A. Schults and I. Taniguchi, The Electrochemical Society, Inc., Pennington, pp. 153-164, 1993.

7. Separation of Hydrogen-bonding Donors in Capillary Electrophoresis Using Polyethers as Matrix.
Yukihiro Esaka, Yasuo Yamaguchi, Kenji Kano, Masashi Goto,
Hiroki Haraguchi and Jun-ichi Takahashi
Anal. Chem., **66** (1994) 2441-2445.
8. Kinetic Analysis of Oxazolopyrroloquinoline Formation in the Reaction of Coenzyme PQQ with Amino Acids by Capillary Zone Electrophoresis.
Yukihiro Esaka, Yasuo Yamaguchi, Masashi Goto and Kenji Kano
J. Chem. Soc. Perkin Trans. 2, **1994**, 2163-2168.
9. Hydrogen-bonding Interaction in Capillary Electrophoresis Using Polyether Matrices.
Yukihiro Esaka, Masashi Goto, Hiroki Haraguchi, Tokuji Ikeda
and Kenji Kano
J. Chromatogr. A, in press.